Research Programs Unit for Molecular Neurology
Faculty of Medicine
Doctoral Programme in Biomedicine
University of Helsinki
Finland

NEW TOOLS FOR MITOCHONDRIAL DISEASE DIAGNOSIS: FGF21, GDF15 AND NEXT-GENERATION SEQUENCING

Jenni M Lehtonen

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Supervised by

Professor Anu Suomalainen Wartiovaara, MD, PhD,
Research Programs Unit, Molecular Neurology, Biomedicum Helsinki, University of Helsinki and Department of Neurology, Helsinki University Central Hospital, Finland

Reviewed by

Docent Mika Martikainen, MD, PhD,
Division of Clinical Neurosciences, University of Turku and Turku University Hospital, Turku, Finland.

Professor Carolyn Sue, MD, PhD.
Department of Neurogenetics, Kolling Institute of Medical Research, University of Sydney and Royal North Shore Hospital, Sydney, Australia; and the Department of Neurology Royal North Shore Hospital, Sydney, Australia

Discussed by

Professor Rita Horvath, MD, PhD,
Institute of Genetic Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom

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To patients, and their families,
with devastating progressive disorders,
without a cure

ABSTRACT

Mitochondrial diseases are inheritable diseases, where the function of the ATP (adenosine triphosphate) producing organelle of the cell, is compromised. This leads to a wide variety of phenotypes, known to arise from defects in over 200 genes. Typically manifesting organs are brain, heart, muscle, liver, endocrine organs and sense organs. Mitochondrial disorders are often progressive, they can manifest in multiple organs in one person and due to inheritance, other family members might also be affected. Careful clinical assessment with investigation of family history helps to predict the cause, but other diagnostic assessments play a central role in diagnosis.

Elevation of blood or cerebrospinal fluid lactate has traditionally been an indicator of mitochondrial dysfunction, but this biomarker lacks sensitivity and specificity, and more accurate biomarkers are required. Muscle biopsy sample is the gold standard of mitochondrial disease diagnosis. Cytochrome c oxidase (COX) negative, succinate dehydrogenase (SDH) positive fibers and ragged-red fibers (RRFs) are hallmarks of mitochondrial dysfunction. Reduced respiratory chain enzyme activity in tissue verifies diagnosis. Solid diagnosis requires genetic evidence, but the expanding number of disease-causing genes makes it difficult to choose which genes to sequence.

We report here a novel diagnostic serum biomarker, fibroblast growth factor 21 (FGF21), which is more sensitive and specific to muscle-manifesting mitochondrial disorders than any of the conventional biomarkers used before. It correlates with COX-negative muscle fibers and is most likely produced and secreted by them. We also studied another recently discovered serum biomarker, growth differentiation factor 15 (GDF15). We report that both FGF21 and GDF15 correctly distinguish mitochondrial myopathies from non-mitochondrial myopathies and controls, making them the most accurate biomarkers for mitochondrial myopathies to date. The trigger for induction of these biomarkers seems to be upstream of respiratory chain defect, most likely initiates from the mitochondrial translational machinery.

In another study, we used next generation sequencing to search for a pathogenic mutation in a patient with fatal infantile Alpers hepatoencephalopathy. We identified two compound heterozygous mutations in a novel disease gene, *FARS2*. This gene encodes for a protein, mitochondrial phenylalanyl-tRNA synthetase (mtPheRS), responsible for the charging of mitochondrial phenylalanyl-tRNA with its cognate amino acid. Structural prediction of the mutated proteins together with functional studies in *E. coli* showing decreased activity of mutant mtPheRS, verified the diagnosis.

Our results strongly support the use of FGF21 and GDF15 as first line diagnostic assessments. Rapid progression to next-generation sequencing is advised if both of these biomarkers are elevated, with positive predictive value being 95%. This would reduce the need for invasive diagnostic tests.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I. Suomalainen,A., **Elo,J.M.***, Pietilainen,K.H.*, Hakonen,A.H., Sevastianova,K., Korpela,M., Isohanni,P., Marjavaara,S.K., Tyni,T., Kiuru-Enari,S., *et al.* (2011) FGF-21 as a biomarker for muscle-manifesting mitochondrial respiratory chain deficiencies: a diagnostic study. *Lancet Neurol*, **10**, 806–818.

II. **Lehtonen,J.M.***, Forsstrom,S.*, Bottani,E., Viscomi,C., Baris,O.R., Isoniemi,H., Hockerstedt,K., Osterlund,P., Hurme,M., Jylhava,J., *et al.* (2016) FGF21 is a biomarker for mitochondrial translation and mtDNA maintenance disorders. *Neurology*, **87**, 2290–2299.

III. **Elo,J.M**., Yadavalli,S.S., Euro,L., Isohanni,P., Gotz,A., Carroll,C.J., Valanne,L., Alkuraya,F.S., Uusimaa,J., Paetau,A., *et al.* (2012) Mitochondrial phenylalanyl-tRNA synthetase mutations underlie fatal infantile Alpers encephalopathy. *Hum Mol Genet*, **21**, 4521–4529.

In addition, some unpublished data are presented.

*equal contribution

ABBREVIATIONS

AARE	amino acid response element
aaRS	aminoacyl-tRNA synthetase
acetyl-Coa	acetyl-coenzyme A
ACMG	American College of Medical Genetics
ADP	adenosine diphosphate
adPEO	autosomal dominant progressive external ophthalmoplegia
ALS	amyotrophic lateral sclerosis
ANOVA	1-way analysis of variance
arPEO	autosomal recessive progressive external ophthalmoplegia
ATF4	activating transcription factor 4
ATP	adenosine triphosphate
AUC	area under curve
BMI	body mass index
BN-PAGE	blue native polyacrylamide gel electrophoresis
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CK	creatine (phospho)kinase
CNS	central nervous system
COPD	chronic obstructive pulmonary disease
CoQ10	coenzyme Q10, ubiquinone
COX	cytochrome c oxidase
CSF	cerebrospinal fluid
CVS	chorionic villus sample
CI	complex I, NADH dehydrogenase
CII	complex II, succinate dehydrogenase
CIII	complex III, CoQ10-cytochrome c oxidoreductase
CIV	complex IV, cytochrome c oxidase
CV	complex V, ATP synthase
dNTP	deoxynucleotide triphosphate
EEG	electroencephalogram
ES cell	embryonic stem cell
$FADH_2$	flavin adenine dinucleotide (reduced form)
FARS2	mitochondrial phenylalanyl-tRNA synthetase, gene
Fe-S	iron-sulphur
FGF21	fibroblast growth factor 21
FIMM	Finnish Institute for Molecular Medicine
GDF15	growth differentiation factor 15
GFM1	translation elongation factor G
GRACILE	growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis and early death
GTP	guanosine triphosphate

HSP	heavy strand promoter
IBM	inclusion body myositis
IF2	mitochondrial translation initiation factor 2
IF3	mitochondrial translation initiation factor 3
IOSCA	infantile-onset spinocerebellar ataxia
iPS	induced pluripotent stem
IQR	interquartile range
KO	knock out
KSS	Kearns-Sayre syndrome
LBSL	leukoencephalopathy with brain stem and spinal cord involvement and high brain lactate
LGMD	limb-girdle muscular dystrophy
LHON	Leber's hereditary optic neuropathy
L/P	lactate to pyruvate ratio
LSP	light strand promoter
LSU	large subunit of mitochondrial ribosome
mCRC	metastasized colorectal cancer
MELAS	mitochondrial encephalomyopathy, lactic acidosis and stroke – like episodes
MIDD	maternally inherited diabetes and deafness
MILS	maternally inherited Leigh syndrome
MIRAS	mitochondrial recessive ataxia syndrome
MM	mitochondrial myopathy
MNGIE	mitochondrial neurogastrointestinal encephalopathy
MRI	magnetic resonance imaging
mRNA	messenger RNA
MRS	magnetic resonance spectroscopy
mtDNA	mitochondrial DNA
MTERF1	mitochondrial transcription termination factor 1
mtPheRS	mitochondrial phenylalanyl-tRNA synthetase
MTRF1	mitochondrial translational release factor 1
NAA	N-acetyl L-aspartate
NADH	nicotinamide adenine dinucleotide (reduced form)
NARP	neurogenic muscle weakness, ataxia and retinitis pigmentosa
nDNA	nuclear DNA
NGS	next generation sequencing
O_H	replication origin of heavy strand
O_L	replication origin of light strand
OXPHOS	oxidative phosphorylation
PBC	primary biliary cirrhosis
PDHD	pyruvate dehydrogenase deficiency
PEO	progressive external ophthalmoplegia
POLG	mitochondrial polymerase gamma
POLMRT	mitochondrial RNA polymerase
PPARα	peroxisome proliferator-activated receptor alpha

PSC	primary sclerosing cholangitis
QF	quadriceps femoris muscle
qPCR	quantitative polymerase chain reaction
RC	respiratory chain
RITOLS	RNA incorporation during mtDNA replication
ROC	receiver operating characteristic
ROS	reactive oxygen species
RRF	ragged-red fiber
rRNA	ribosomal RNA
SD	standard deviation
SDH	succinate dehydrogenase, CII
SM	statin-induced myopathy
SMA	spinal muscular atrophy
SNV	single nucleotide variant
SSBP	single-stranded DNA-binding protein
SSU	small subunit of mitochondrial ribosome
TCA cycle	tricarboxylic acid cycle
TFAM	transcription factor A
TFB1M	mitochondrial transcription factor 1
TFB2M	mitochondrial transcription factor 2
TGF- β	transforming growth factor beta
TK2	thymidine kinase 2, gene
tRNA	transfer RNA
TUFM	translation elongation factor Tu
TWINKLE	mitochondrial DNA helicase
VUS	variant of unknown significance
WES	whole exome sequencing
ΔmtDNA	single large-scale mtDNA deletion
2D-AGE	two-dimensional agarose gel electrophoresis

1 INTRODUCTION

Mitochondria are the ATP-producing organelles of the cell. Dysfunction of mitochondrial ATP producing system, the respiratory chain (RC) and the ATP synthase, is the most common error of inborn metabolism (1). These disorders can manifest in nearly any organ of the body, in both children and adults. Typically, brain, muscle, liver, endocrine organs and/or sense organs are affected, sometimes with distinct combinations of symptoms. Severity ranges from fatal pediatric encephalopathies to mild restricted muscular dysfunction manifesting only in late adulthood.

The RC and ATP synthase consist of more than 90 protein subunits encoded by both mitochondrial and nuclear DNA (nDNA). The first mitochondrial RC dysfunction causing mutations were found in the mitochondrial genome (mtDNA) in 1988, but since then, the number of known genetic defects in both genomes has grown tremendously (2). To date, more than 200 genes are known to cause a mitochondrial disorder.

Diagnosis of these disorders is challenging due to the great variety of symptoms and their severity, tissue specificity of pathology, the many inheritance models possible and the varying penetrance of some diseases (3). Diagnosis begins with careful investigation of family history and clinical assessment of a patient followed by biochemical analyses of blood, urine and/or cerebrospinal fluid, tissue biopsy, imaging, electrophysiological examination and gene sequencing (4). Definitive diagnosis requires genetic diagnosis or an obvious defect in tissue mitochondrial RC function.

This thesis summarizes the diagnostic assessments currently used and the advances our research has given to this field. We show how novel serum biomarkers (FGF21 and GDF15) perform in diagnosis and what the likely trigger for their induction is. We also show how next generation sequencing (NGS) can be a powerful tool when searching for mutations in novel disease causing genes for mitochondrial disorders.

2 REVIEW OF THE LITERATURE

2.1 MITOCHONDRION AS AN ORGANELLE

Mitochondria are intra-cellular organelles that have a distinct evolutionary origin. It is thought that an archaebacterium once engulfed an aerobic proteobacterium achieving greater capacity for aerobic respiration and thus a head start for cell survival (5). Over time, evolution has modified both the archaebacterium and the proteobacterium (now, mitochondrion) but some features still link the latter to a bacterium. First, human mtDNA is circular, instead of linear. The size of the genome used to be much larger, but most content has been incorporated to the host cell's genome (6). Second, translation of mitochondrial proteins requires machinery that is similar to that of bacteria (7). This is of clinical relevance, while some antibiotics (aminoglycosides, tetracyclines) that are developed to prevent bacterial growth (more precisely, protein translation), also affect mitochondrial translation with considerable side effects (aminoglycoside-induced deafness) (8). Each mitochondrion is surrounded by a double membrane, of which the inner is highly invaginated, possibly to increase surface area for the mitochondrial respiratory chain (9). Inside the inner membrane is the mitochondrial matrix.

2.1.1 MITOCHONDRIAL GENETICS AND PROTEIN SYNTHESIS

Human mitochondrial DNA consists of 16,569 base pairs comprising 37 genes on two strands without introns. These genes contain the instructions for the making of 13 mitochondrial proteins, 22 transfer RNAs (tRNA) and two ribosomal RNAs (rRNA) (10), of which the RNAs are needed to produce the proteins (Figure 1). The 13 proteins are subunits of the respiratory chain complexes CI, CIII, CIV, as well as the ATP synthase (CV). A total of 93 proteins are needed to build complexes I-V, the rest of them being encoded by the nuclear DNA. Complex II is the only RC complex consisting solely of nDNA encoded proteins.

Every eukaryotic cell has numerous mitochondria (up to 100 000 in an ovum (11)), and every mitochondrion has several copies of mtDNA, with copy number typically being 2-10 per mitochondrion (10,11). MtDNA is covered with protein (mostly transcription factor A, TFAM) and packed into nucleoids that are attached to the inner mitochondrial membrane. TFAM is also known to regulate mtDNA copy number (14).

The replication machinery needed for copying mtDNA *in vitro* is encoded by the nucleus and is very simple, consisting only of three proteins: the mitochondrial DNA polymerase gamma (POLG), single-stranded DNA-binding

protein (SSBP) and mitochondrial helicase TWINKLE (15). Unlike nuclear DNA, replication of mtDNA is independent of cell cycle (16, 17).

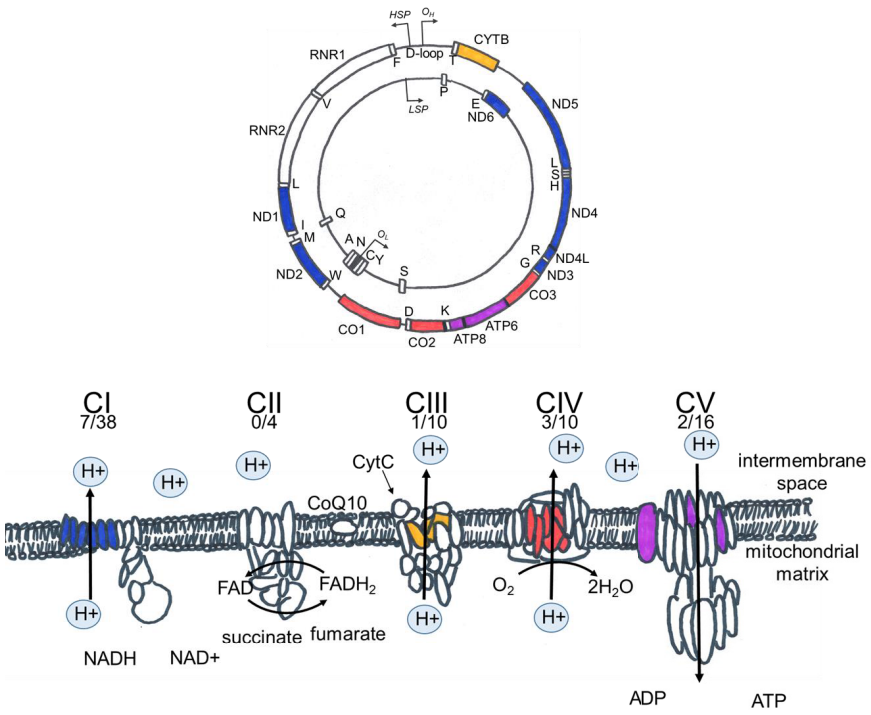


Figure 1. Mitochondrial DNA (mtDNA) and oxidative phosphorylation (OXPHOS). MtDNA encodes a minority of OXPHOS system (CI-CV) subunits, indicated as numbers of mtDNA encoded/nuclear encoded subunits above each complex. Colors in mtDNA indicate the complex the protein belongs to. Complex II is the only complex encoded fully by the nuclear DNA and it is also an enzyme of the TCA cycle. CI, CIII and CIV pump protons across the inner membrane to create an electrochemical force, which drives the synthesis of ATP by CV. NADH and $FADH_2$ (products of TCA cycle) bring electrons (not shown) to CI and CII which transfer them to CoQ10-> CIII->CytC. Cyt C carries these electrons to CIV and molecular oxygen (O_2) to produce water (H_2O). Single alphabets in mtDNA indicate tRNAs: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; Q, glutamine; P, proline; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. HSP, transcription promoter of the heavy strand; LSP, transcription promoter of the light strand; O_H, replication origin of the heavy strand; O_L, replication origin of the light strand. H+, proton; CytC, cytochrome c; CoQ10, coenzyme Q10. HGNC approved gene symbols are used. Illustration: Kustaa Lehtonen

There are several models of mitochondrial DNA replication, the strand displacement model being the first described (16, 18). In this model, replication is initiated at the heavy strand replication of origin (O_H) after mitochondrial RNA polymerase (POLMRT) has synthetized a short RNA primer. The

replication machinery begins synthesis of complementary DNA from this primer, leaving the other strand (heavy strand) aside. It has been proposed that this strand is covered with SSBP probably to protect it from uncontrolled binding of the replicative enzymes or to mediate the contact between TWINKLE and POLG (19). Replication of the leading strand continues about two thirds of the full mtDNA circle, until it reaches the replication origin of the light strand (O_L) (Figure 1). This O_L is displaced from light (leading) strand and it forms a new stem-loop structure, which promotes initiation of the lagging strand DNA synthesis in the opposite direction. Both strands are then synthetized simultaneously until two copies of mtDNA are ready and separated from each other. Replication can proceed multiple rounds in a row. Often, at the beginning, leading strand replication is terminated soon after O_H forming a triple stranded region of about 650bp, called the D-loop (18). Function of this loop is unknown, but hypotheses suggesting a role in mtDNA synthesis regulation and recruitment of replication machinery have been postulated (18) (Figure 1).

Later, analysis of mtDNA from highly purified mitochondria with two-dimensional agarose gel electrophoresis (2D-AGE) suggested another replication model, RNA incorporation during mtDNA replication (RITOLS). In the 2D-AGE, mtDNA replication intermediates form arcs based on their masses. Treating the mtDNA with restriction enzymes that only digest one or two stranded RNA or DNA revealed unexpected changes in 2D-AGE arcs, suggesting that some of the lagging strand is bound to complementary RNA instead of protein. In RITOLS, there are two replication origins instead of only one, both of which are in the D-loop area. (20)

Synthesis of mitochondrially encoded proteins requires transcription of mtDNA by POLRMT together with TFAM and transcription factor B2 (TFB2M) or B1 (TFB1M, less active) (21). Transcription begins at the light strand promoter (LSP) and the heavy strand promoter (HSP) in the light and heavy strands respectively. Transcription is terminated by mitochondrial transcription termination factor MTERF1 (22) and the resulting messenger RNA (mRNA) is polycistronic, containing the instructions for multiple genes in one strand. Messenger RNA is processed by specific enzymes to cleave individual genes, tRNAs and rRNAs which are further modified for example by polyadenylation, or fold into correct secondary and tertiary structure (23).

Next, in a process called translation, this mRNA functions as a scaffold for tRNAs in the presence of the mitochondrial ribosome (mitoribosome) and protein is formed from tRNA-bound amino acids. Amino acids are attached to the acceptor stem of cognate tRNAs by aminoacyl-tRNA synthetases (aaRSs). Translation is initiated by mitochondrial translation initiation factor 3 (IF3) that helps mRNA to bind to the small ribosomal subunit (SSU) (23). Translation initiation factor 2 (IF2) helps formylated $tRNA^{Met}$ (the first tRNA) to bind SSU and the start codon in mRNA. The large ribosomal subunit (LSU) is attached to the complex while IF2 and IF3 are released. In translation elongation, mRNA moves through mitoribosome while it is read three nucleotides (a codon) at a

time. Translation elongation factor Tu (TUFM) binds to aminoacylated tRNA (the second tRNA) and after proofreading, carries it to the mitoribosome, where tRNA is recognized by the anticodon site and TUFM is released. Adjacent tRNA-bound amino acids are linked to each other and nascent polypeptide exits the mitoribosome. Translation elongation factor G (GFM1) catalyzes the translocation of these tRNAs to the next positions in the mitoribosome while it moves one codon forward. A third tRNA enters while the first tRNA exits the mitoribosome (Figure 2). This elongation process is repeated until one of the stop codons of mRNA is reached. Stop codons are recognized by translational release factor RF1 (MTRF1), which causes the ready polypeptide chain to be released from the tRNA for further modifications. Translation factors and ribosomal subunits are recycled for another round of translation. The whole process requires energy in the form of guanosine triphosphate, GTP (23).

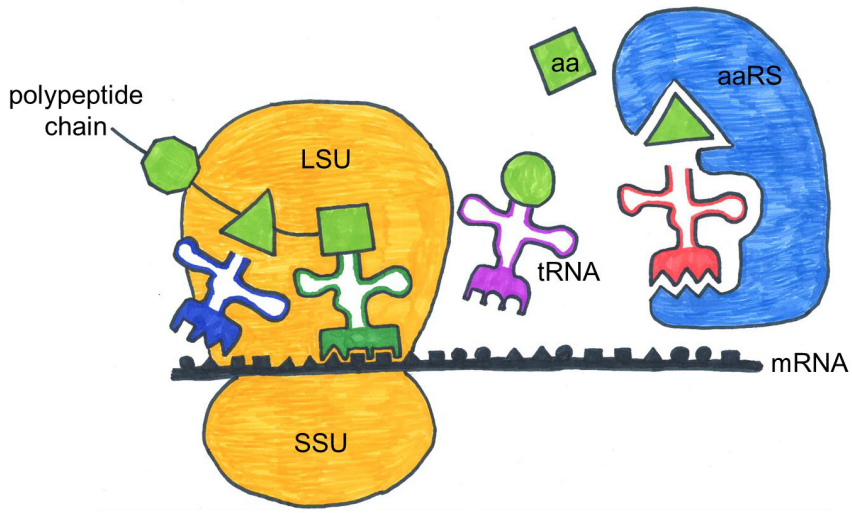


Figure 2. Simplified illustration of mitochondrial translation. Aminoacyl-tRNA synthetase (blue) catalyzes the attachment of amino acid (green) to its cognate tRNA. While mitochondrial ribosome (orange) moves along mRNA, tRNAs bind to it according to codon sequence. tRNA- bound amino acids are attached to each other by covalent bond, forming polypeptide chain that exits the ribosome. The polypeptide chain is further modified to produce a fully functioning protein. LSU, large subunit; SSU, small subunit; tRNA, transfer RNA; mRNA, messenger RNA; aa, amino acid; aaRS, aminoacyl-tRNA synthetase. Illustration: Kustaa Lehtonen

2.1.2 ATP PRODUCTION BY OXIDATIVE PHOSPHORYLATION

Mitochondria are the sites where dietary energy is converted to ATP in the process of oxidative phosphorylation (OXPHOS). This procedure requires oxygen and is highly efficient: it produces 32 units of energy per glucose molecule while anaerobic glycolysis produces only two.

First, dietary carbohydrates, protein and triglycerides are metabolized by specific enzymes to produce acetyl-coenzyme A (acetyl-CoA). This metabolite then enters the mitochondrial citric acid cycle (TCA cycle, Kreb's cycle) (24), where it is further metabolized in multiple steps. These reactions produce (among others) reducing equivalents NADH (nicotinamide adenine dinucleotide) and $FADH_2$ (flavin adenine dinucleotide), which are needed as electron donors in the electron transport chain located in the inner mitochondrial membrane.

The electron transport chain consists of four complexes. Complex I (NADH dehydrogenase) oxidizes NADH and shuttles electrons to coenzyme Q10 (CoQ10, ubiquinone). Complex II is also a TCA cycle enzyme, succinate dehydrogenase, which converts succinate to fumarate yielding $FADH_2$. Similar to NADH, $FADH_2$ donates electrons to CoQ10. Oxidized NADH (NAD+) and $FADH_2$ (FAD) are recycled back to TCA cycle. CoQ10 moves along the inner membrane, carrying the electrons from CI and CII to complex III (CoQ10-cytochrome c oxidoreductase). This complex subsequently moves the electrons from oxidized CoQ10 (ubiquinol) to cytochrome c, a water-soluble electron carrier of the intermembrane space. Finally, the electrons are donated to molecular oxygen (O_2) in complex CIV (cytochrome c oxidase), producing water (H_2O). Complexes I, III and IV also pump protons (H+) across the inner mitochondrial membrane creating an electrochemical gradient, which is utilized by complex V (ATP synthase) in a reaction where ADP (adenosine diphosphate) is phosphorylated to ATP (Figure 1). (25, 26)

Besides nuclear and mitochondrial-encoded subunits of RC, proper function of the complexes requires additional nuclear encoded assembly factors (Table 1). Complexes form dimers and polymers and even larger supercomplexes, where different complexes are associated with each other in a functional unit. Complex II is the only complex not found in these supercomplexes. ATP synthase is a dimer, often found on the curvatures of the inner mitochondrial membrane, whereas the other complexes are located on the flat area. Tight connection of complexes is thought to prevent formation of free radicals as leakage of electrons from the RC is minimized when electrons are shuttled from one complex to another in close proximity. Complexes are shown to be more stable in supercomplexes than alone yet a mutation in one complex subunit might also affect the stability of another complex. (26)

Occasional electron leakage from the RC happens, creating reactive oxygen species (ROS). These free radicals are important signaling molecules, but toxic in large quantities. Uncontrollable production of ROS causes mutagenesis and cellular damage resulting in mitochondrial dysfunction, tumor formation, ageing, apoptosis and necrosis. (27)

2.1.3 OTHER FUNCTIONS OF MITOCHONDRIA

Despite cells' high requirement for ATP, OXPHOS is not the only important function of mitochondria. Under some conditions, cells survive without mitochondrially produced ATP, but never does a eukaryotic cell remain viable without iron-sulphur (Fe-S) cluster dependent protein (28). These clusters are synthesized in mitochondria and some are exported to the cytosol. Fe-S containing proteins are essential for nuclear genome maintenance, gene expression and stability (28).

Folate (vitamin B_9) cycle partially localizes to mitochondria, using serine, glycine and sarcosine to produce, among others, formyl-methionine needed for mitochondrial translation initiation (29). Fatty acids enter mitochondria by carnitine shuttle as acyl-CoA esters, which are metabolized in the mitochondrial matrix by beta-oxidation to acetyl-CoA, which enters the TCA cycle.

In addition, mitochondria participate in various cellular processes such as heme and biotin (vitamin B_7) synthesis, calcium storage and programmed cell death (30).

2.2 MITOCHONDRIAL DISORDERS

Mitochondrial diseases are the most common inborn errors of metabolism with a very heterogeneous genetic aetiology resulting in a wide spectrum of phenotypes (Table 1). Currently 1200-1300 genes are known to encode a mitochondria-linked protein (most of them listed in MitoCarta 2.0 (31, 32)), while only 250-300 of them are known to cause disease (The Mitochondrial Disease Sequence Data Resource Consortium (MSeqDR, https://mseqdr.org/)). Only a proportion of these, however, primarily affect the mitochondrial RC, causing primary OXPHOS disorders, the focus of this thesis (Table 2). The prevalence of primary OXPHOS disorders is estimated as 1/2000- 1/10 000 live births (33–37).

Mortality in pediatric mitochondrial disorders is 10-50% per year after diagnosis and 5-20% per year after clinical onset of symptoms in adulthood (35). However, prognosis depends greatly on the genotype and phenotype: severe early-onset encephalopathies (Leigh, Alpers) naturally have a poor prognosis (38, 39) as compared to isolated muscle weakness of the external ophthalmic muscles (progressive external ophthalmoplegia, PEO). Early diagnosis and treatment of both the primary disease and its complications is of uttermost importance for better outcome.

Table 1. *Examples of relevant primary OXPHOS disorders, their genetic background and typical symptoms. * indicates a group of three disorders (PEO, Pearson, KSS) forming a disease entity with varying severity. Patients who survive the anemia of Pearson syndrome tend to develop KSS later. HGNC approved gene symbols are used.*

Disorder	Full name	Symptoms	Gene defect	Inheritance	Reference
Alpers	diffuse degeneration of cerebral gray matter (with hepatic cirrhosis)	intractable seizures, developmental delay, liver disease	typically POLG, but also TWNK, MTCO2, FARS2, PARS2, NARS2	recessive	Hakonen et al 2008, Uusimaa et al 2003, Elo et al 2012, Sofou et al 2015
GRACILE	growth retardation, amino aciduria, cholestasis, iron overload, lactic acidosis and early death	growth retardation, failure to thrive	BCS1L	recessive	Visapää et al 2002
IOSCA	infantile-onset spinocerebellar ataxia	ataxia, hypotonia, athetosis, epilepsy, ophthalmoplegia, sensorineural deafness, peripheral neuropathy	TWNK	recessive	Nikali et al 2015
PEO*	progressive external ophthalmoplegia	muscle weakness, progressive external ophthalmoplegia, ptosis, exercise intolerance	sporadic ΔmtDNA, occasionally partial duplication of mtDNA	sporadic	Yamashita et al 2008
Pearson syndrome*	sideroblastic anemia with marrow cell vacuolization and exocrine pancreatic dysfunction	megaloblastic anemia, renal insufficiency, dysfunction of exocrine pancrease, growth retardation			Rötig et al 1995
Kearns-Sayre syndrome, KSS*	ophthalmoplegia, pigmentary degeneration of retina and cardiomyopathy	progressive external ophthalmoplegia, retinitis pigmentosa, heart conduction defect			Zeviani et al 1998
Leigh	subacute necrotizing infantile encephalopathy	motor or intellectual retardation, brainstem dysfunction (nystagmus, ophthalmoparesis, respiratory abnormalities), ataxia, dystonia, optic atrophy	over 75 causative genes reported, typically affecting CI or mtDNA translation	autosomal recessive or maternal	Lake et al 2016
LHON	Leber's hereditary optic neuropathy	painless acute or subacute visual loss during second or third decade of life	several mtDNA mutations including CI subunits, typically high heteroplasmy, male predominance	maternal	Yu-Wai-Man et al 2011, Hudson G et al 2007
MERRF	myoclonic epilepsy associated with ragged-red fibers	myoclonic epilepsy, ataxia, muscle weakness, cardiomyopathy, lipomatosis	m.8344A>G (MTTK) in 80-90% of cases, other tRNAs	maternal	Shoffner and Wallace 1992
MELAS, MIDD	mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), maternally inherited diabetes and deafness (MIDD)	muscle weakness, stroke-like symptoms, epilepsy, cardiomyopathy (MELAS), sensorineural hearing loss, diabetes mellitus (MIDD)	m.3243A>G (MTTL1) in 80% of cases, other tRNAs and mtDNA rearrangements	maternal	Goto 1990, Maassen JA and Kadowaki T 1996
ANS	ataxia neuropathy spectrum (ANS) including: mitochondrial recessive ataxia syndrome (MIRAS), sensory ataxia, neuropathy, dysarthria and ophthalmoplegia (SANDO) and spino-cerebellar ataxia epilepsy syndrome (SCAE)	ataxia, epilepsy, myoclonus, nystagmus, dysarthria, sensorimotor neuropathy, mild cognitive impairment, ophthalmoplegia	POLG	recessive	Hakonen et al 2005, Schulte C 2009, Tzoulis et al 2006
MEMSA	myoclonic epilepsy, myopathy, sensory ataxia	epilepsy, myopathy, ataxia, no ophthalmoparesis	POLG	recessive	Van Goethem et al 2004
MNGIE	mitochondrial neurogastrointestinal encephalopathy	cachexia, leukoencephalopathy, peripheral neuropathy, ptosis, ophthalmoparesis	TYMP, RRM2B, POLG	recessive	Gamez et al 2002, Shaibani et al 2009, Tang et al 2012
NARP	neuropathy, ataxia and retinitis pigmentosa	sensory neuropathy, ataxia, retinitis pigmentosa, mental retardation, poor night vision, Leigh	most often MTATP6	maternal	Holt et al 1990
PEO	progressive external ophthalmoplegia	muscle weakness, progressive external ophthalmoplegia, ptosis, exercise intolerance	AFG3L2, C20orf7, DGUOK, DNA2, DNM2, MPV17, OPA1, POLG, POLG2, RNASEH1, RRM2B, TWNK, TYMP, SLC25A4, SPG7, TK2	autosomal dominant or recessive	Gorman et al 2016

2.2.1 GENETICS OF PRIMARY OXPHOS DISORDERS

Primary OXPHOS disease genes encode for proteins that are closely linked to OXPHOS complexes. Proteins affecting mtDNA replication and maintenance or synthesis of deoxynucleotide triphosphates (dNTPs) cause point mutations or deletions in mtDNA and/or a reduction in mtDNA quantity (depletion). Translation of mtDNA-encoded proteins is impaired when the defect affects rRNA, tRNA, tRNA modification, aminoacylation of tRNAs, mRNA processing, ribosomal function or translation factors. Defects in RC subunits or their assembly factors have a direct impact on respiratory chain structure and function. Single large-scale mtDNA deletions (ΔmtDNA) cause OXPHOS deficiency by affecting multiple mtDNA-encoded proteins/RNA at the same time. It is estimated that 75% of adult mitochondrial disorders are caused by mutated mtDNA, whereas in children the proportion is more modest, 10-25% (40).

Some of the disease-causing mutations arise *de novo*, meaning the mutation took place during or right after fertilization of this individual. This mutation is not likely to occur again in the siblings and it is not present in the parents. These mutations are dominant and have been hard to find due to large quantity of heterozygous variants in the genome. Recent development in sequencing technologies has enabled the finding of these as well (41), although not yet in primary OXPHOS disorders (42). More frequent is that the mutation is passed on from one generation to the next in autosomal recessive, autosomal dominant, X-linked or maternal manner. In this case, the likelihood of a person to manifest with a disease depends on the inheritance model, penetrance and in the case of mtDNA mutations, the heteroplasmy of the mutant DNA (see below).

Variant frequency in different populations is affected by population genetics, with the founder effect and the bottleneck phenomenon having an important role. GRACILE syndrome (growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis, and early death) is a disorder belonging to the Finnish disease heritage with one founder mutation (43). It is caused by a mutation in *BCS1L*, encoding for a protein involved in CIII assembly. Similarly, in patients with myopathic mitochondrial depletion syndrome caused by either of the two homozygous *TK2* mutations, a Finnish and possibly a Scandinavian founder were implicated (44).

2.2.1.1 Mitochondrial DNA: maternal inheritance

All eukaryotic cells, except mature erythrocytes, have mitochondria. After fertilization of an egg, the sperm cell mitochondria, however, are actively degraded in the zygote. Because of this, the mitochondrial genome is solely maternally inherited making it impossible for fathers to pass on pathogenic mtDNA to their offspring (with rare exception (45)).

Mitochondrial DNA defects can vary from single nucleotide variations (SNVs) to larger scale deletions, the most common ΔmtDNA being 4.9kb in size. Large deletions of mtDNA are not transmitted to offspring (with few rare exceptions (46, 47)). As healthy (wild type) mtDNA is typically homoplasmic, mutations in mtDNA are usually heteroplasmic, meaning there is also normal mtDNA in the cells. The level of mutated mtDNA, called the heteroplasmy, affects the clinical presentation of the disease, with high variation. Mutations in mitochondrial CV subunit gene *MTATP6* (mitochondrial ATP synthase subunit 6) cause neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP) or maternally inherited Leigh syndrome (MILS) depending on the heteroplasmy level. Having <40% of this mutated mtDNA typically does not cause a disease, but >90% causes early onset fatal encephalopathy (MILS). In contrast, a fairly common mtDNA point mutation, m.3243A>G, causes a wide spectrum of clinical manifestations ranging from maternally inherited diabetes and deafness (MIDD) to mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), but the presentation is not attributed to the heteroplasmy level (48). Some mutations may occur as homoplasmic, as in Leber's hereditary optic neuropathy (LHON) (49), but not all LHON mutation carriers manifest the disease (due to varying penetrance, male predominance). Environmental factors (smoking, alcohol usage, low fruit consumption) are associated with disease expression, but the causal relationship of these two is unknown (49). Association of mtDNA haplogroup with disease manifestation has also been reported (50).

Depletion of mtDNA is a consequence of mutations affecting mtDNA replication and maintenance. Genes that are responsible for mtDNA replication and maintenance (*POLG, TWNK, TK2, RRM2B, TYMP, DGUOK*) cause depletion in addition to mtDNA mutagenesis (51). Depletion causes incapability to express and synthesize sufficient amount of mtDNA encoded products, resulting in an OXPHOS defect (51) (Table 1).

Despite maternal inheritance, a mother with an mtDNA disease can still have healthy babies spontaneously (see also treatment section chapter 2.2.4.3). After egg fertilization, mitochondria are distributed to daughter cells without mtDNA replication, resulting in a decrease in mtDNA copy number per cell. Segregation of mtDNA is thought to happen in a controlled fashion, all daughters (stem cells of developing tissues) being equal in heteroplasmy (52). After numerous cell divisions, primordial germ cells however, have such a small number of mtDNA copies (possibly even only 10 copies (52)) that a small variation in the absolute mutant mtDNA copy number is proportionally large (Figure 3). This is called the bottleneck of mtDNA segregation. During further development of female primordial germ cells (oogenesis), copy number will increase again at least 2000 fold (52) resulting in a wide range in mtDNA heteroplasmy of mature eggs. Thus, a mother with a low heteroplasmy and no or only mild clinical manifestation, can have eggs with 0-100% of mutated mtDNA.

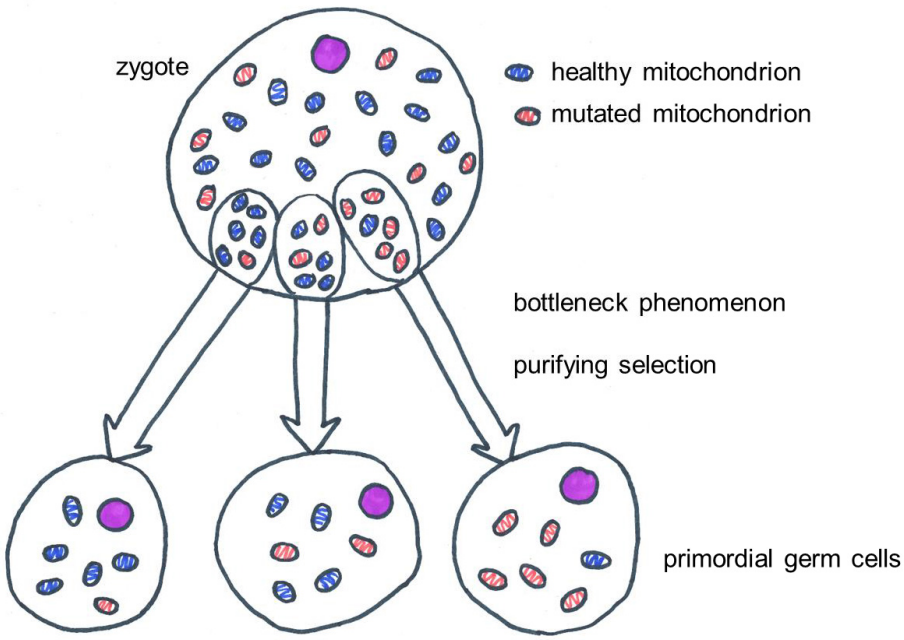
Table 2. *Listed are genes (and ΔmtDNA) known to cause a primary OXPHOS deficiency. Genes are categorized to four groups, first three of them being the same as in paper II. Asterisk indicates genes that also cause mtDNA depletion. Genes are taken from mSeqDR database (https://mseqdr.org/), Gorman GS et al 2016 and Stiles et al 2016.*

Translation	rRNA	MTRNR1
	tRNA	22 mtDNA encoded tRNAs
	aminoacyl tRNA synthetases	AARS2, CARS2, DARS2, EARS2, FARS2, HARS2, IARS2, LARS2, MARS2, NARS2, PARS2, RARS2, SARS2, TARS2, YARS2, VARS2, KARS, GARS, WARS2
	tRNA modification	ELAC2, GTPBP3, HSD17B10, MTFMT, MTO1, TRIT1, TRMT5, TRMU, PUS1
	mRNA modification	LRPPRC, MTPAP, PNPT1
	ribosomal function	MRPL3, MRPL12, MRPL44, MRPS7, MRPS16, MRPS22
	translation factors	AFG3L2, C12orf65, GFM1, GFM2, RMND1, SPG7, TACO1, TUFM, TSFM
mtDNA deletions	nucleoside pools	DGUOK*, TK2*, TYMP*
	mtDNA replication	DNA2, POLG*, POLG2, TWNK*
	mtDNA maintenance	MGME1, RNASEH1, RRM2B*
	ATP/ADP transport	SLC25A4
	mtDNA defect	ΔmtDNA
RC structure& assembly	CI subunits	MTND1, MTND2, MTND3, MTND4, MTND4L, MTND5, MTND6, NDUFA1, NDUFA2, NDUFA9, NDUFA10, NDUFA11, NDUFA12, NDUFA13, NDUFAF6, NDUFB3, NDUFB9, NDUFB11, NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFS6, NDUFS7, NDUFS8, NDUFV1, NDUFV2
	CI assembly	FOXRED1, NDUFAF1, NDUFAF2, NDUFAF3, NDUFAF4, NDUFAF5, NUBPL
	CII subunits	SDHA, SDHB, SDHD
	CII assembly	SDHAF1, SDHAF2
	CIII subunits	LYRM7, MTCYB, TTC19, UQCC2, UQCC3, UQCRB, UQCRC2, UQCRQ
	CIII assembly	BCS1L, HCCS
	CIV subunits	MTCO1, MTCO2, MTCO3, COX412, COX6B1
	CIV assembly	COA5, COX10, COX14, COX15, COX20, PET100, SCO1, SCO2, SURF1
	CV subunits	ATP5A1, ATP5E, MTATP6, MTATP8
	CV assembly	ATPAF2, TMEM70
	CoQ10 synthesis	COQ2, COQ4, COQ6, COQ8A, COQ8B, COQ9, PDSS1, PDSS2
	Cytochrome c	CYC1, CYCS
mtDNA depletion	mtDNA maintenance	TFAM
	TCA cycle	SUCLA2, SUCLG1
	function unknown	MPV17

Abbreviations: rRNA, ribosomal RNA; tRNA, transfer RNA; CI-CV,OXPHOS complexes I to V; CoQ10, coenzyme Q10; TCA cycle, tricarboxylic acid cycle; ΔmtDNA, single large scale deletion of mtDNA. HGNC approved gene symbols are used, where prexif "MT" refers to mtDNA origin.

There seems to be purifying selection against pathogenic mutations in mtDNA as nonsynonymous changes appear to be less frequent in protein-coding regions than synonymous (53). Still, in the case of *MTATP6* mutation m.T8993G, segregation seems to favor the mutated form (54–59). Random segregation of mtDNA happens after organ development, when cells within tissues have different levels of heteroplasmy. This is seen in muscle histology of mitochondrial disease patients, where COX-negative fibers are shown to have a higher heteroplasmy than the COX-positive fibers next to them (60).

Figure 3. Uneven segregation of mtDNA from zygote to primordial germ cells due to the bottleneck phenomenon. Purifying selection further modifies the heteroplasmy of daughter cells. Illustration: Kustaa Lehtonen



2.2.1.2 Nuclear DNA: recessive, dominant and X-linked inheritance

Nuclear genes are inherited from father and mother, one copy of each chromosome from each parent, except the sex chromosomes. In some diseases it is sufficient to have one copy of a defective gene (dominant inheritance), whereas in some, two defective copies are needed (recessive inheritance). In the former, one of the parents has the same disease as child (unless mutation has occurred *de novo*), whereas in the latter, both parents are heterozygous carriers of the mutation without clinical manifestation. The two alleles of the gene can have the same (homozygous) or different

(compound heterozygous) mutation. Examples of recessive diseases include Alpers, mitochondrial recessive ataxia syndrome (MIRAS) and autosomal recessive PEO (arPEO). An example of autosomal dominant disease is autosomal dominant PEO (adPEO) (Table 1).

X-linked disorders are typically recessive and thus only manifest in men. These disorders are inherited from mother (a carrier) to son, while daughters have a 50% chance to become carriers. Manifesting fathers pass on this chromosome to their daughters, who become carriers, but sons only inherit Y-chromosome from father. An example of an X-linked mitochondrial disorder (not a primary OXPHOS disorder) is Barth syndrome, affecting tafazzin gene (*TAZ*) causing cardiomyopathy, myopathy, neutropenia, 3-methylglutaconic aciduria and poor growth (61).

2.2.2 PHENOTYPE

Mitochondrial diseases cause a wide spectrum of manifestations with a great variation in clinical presentation even with the same underlying mutation (62). Multi-tissue presentation of symptoms, varying phenotype within a family and progressive nature of disease are typical for mitochondrial disorders (63).

Mitochondrial disorders can manifest in newborns or later in life. In a cohort of 100 pediatric mitochondrial disease patients, 80% of them manifested before the age of two (64). Presumably a portion of fetal deaths is explained by severe mitochondrial dysfunction, since despite the existence of heterozygous carriers, some mutations are never found as homozygotes (65, 66).

It is typical that an environmental factor, such as illness, infection, surgery, prolonged fasting or medication elicits deterioration of symptoms in mitochondrial disease (67). Examples of medication-induced mitochondrial dysfunctions are valproate induced liver failure (typical in patients with *POLG* mutations (68)) and aminoglycoside induced hearing loss (8).

Mitochondrial diseases can be very tissue specific (muscle tissue in *TK2* mutations (69) and brain in *DARS2* mutations (65, 70)) and even cell-type specific (retinal ganglion cells in LHON (71), pancreatic beta cells in MIDD), but also multi-organ manifestation in distinct combinations are common. Listed in Table 1 are the most common primary OXPHOS disorders, their genetic background and typical symptoms.

One of the severe childhood-onset mitochondrial disorders is Alpers syndrome (or Alpers-Huttenlocher syndrome, used in case of additional liver manifestation). The disorder was originally described by Bernard Alpers, who discovered the typical neuropathological findings in the cerebral, and sometimes cerebellar cortices, and thalamus: necrosis (microcystic degeneration, capillary proliferation), neuronal loss (gliosis) and spongiosis (39, 72). Involvement of calcarine and striate cortices presents as blindness. It is a disorder causing (hepato) cerebral mtDNA depletion, due to recessive (homozygous or compound heterozygous) *POLG* (71, 37), *TWNK* (74) or

MTCO2 (cytochrome c oxidase subunit II) mutations (75). Age of onset is typically between two and four years of age. The diagnostic triad of this disorder is developmental delay, intractable seizures, and liver failure (Alpers-Huttenlocher syndrome). Additional diagnostic symptoms include cortical blindness and optic atrophy. Valproic acid is liver-toxic for Alpers patients (76). Abnormal diagnostic findings are reduced N-acetyl L-aspartate (NAA) in magnetic resonance spectroscopy (MRS), elevated blood and cerebrospinal fluid (CSF) lactate and CSF protein, abnormal activity in electroencephalogram (EEG), decreased POLG activity and isolated defect in CIV or combined defect in CI, CIII and CIV activities. Neuropathological findings include neuronal loss, spongiform degeneration and astrocytosis of the cortex. Liver shows steatosis and cirrhosis. This disorder is progressive, leading to fatal encephalopathy or liver failure and often death within four years after first symptoms.(39)

2.2.3 DIAGNOSIS OF MITOCHONDRIAL DISORDERS

Careful clinical assessment includes anamnestic interview with investigation of family history. Family members might have a very different phenotype (77–79), and thus active family tracing and examination of family members is informative.

There are multiple disease scoring systems, for both adults and children, which help to determine whether a disease is of mitochondrial aetiology (80–82). Symptoms and findings are scored and total score reflects the likelihood or a mitochondrial disorder ranging from unlikely to definite. A typical diagnostic pathway is represented in Figure 4.

2.2.3.1 Biochemical analyses

Elevated lactate is a typical hallmark of mitochondrial disorders. Lactate is produced from pyruvate, which is normally oxidized to acetyl-coA, which enters the TCA cycle. However, when OXPHOS is impaired, pyruvate is reduced to lactate to produce ATP. This increases the lactate to pyruvate ratio (L/P), a surrogate measure of cytoplasmic NADH/NAD+ ratio. A study by Debray FG et al showed that when lactate is >5mmol/l, high L/P (>25.8) correctly distinguishes mitochondrial diseases (in this study, MELAS, KSS (Kearns-Sayre syndrome) and mitochondrial myopathy with ragged red fibers) from pyruvate dehydrogenase deficiency (PDHD) (83). Lactate and pyruvate can also be measured in CSF. Compared to blood, CSF lactate is less prone to false positivity, but such lack of specificity exists due to central nervous system tumor, inflammation, infection, stroke or seizure (84) and thus should be measured in a chronic steady state of disease. Repeated lactate measurements are used in treadmill or bicycle ergometry to reveal inadequate oxygen utilization or impaired lactate removal.

Creatine (phospho) kinase (CK) is an enzyme abundant in muscle tissue, and it catalyzes the conversion of creatine to phosphocreatine, another high-energy phosphate molecule besides ATP. Serum CK is used in the diagnosis of muscular dystrophies (85) and thus helps in differential diagnosis.

Elevated plasma alanine is occasionally detected in long-standing pyruvate overload, and it is indicative for a mitochondrial disease (4). Elevated plasma or urine amino acids, organic acids, (acyl) carnitine, CoQ10, ammonia and TCA cycle intermediates are also used in (differential) diagnosis, all suggestive for a defect in a particular cellular pathway. (4, 86)

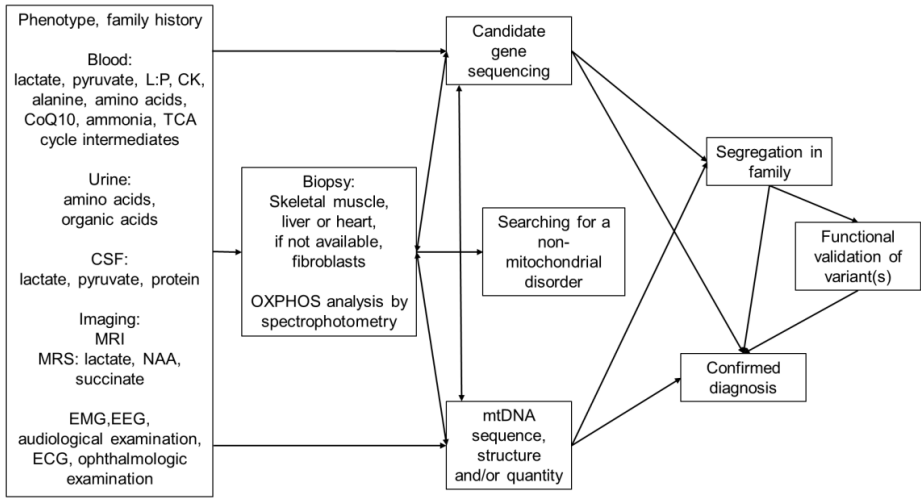


Figure 4. Typical diagnostic pathway for mitochondrial disorders. Abbreviations: L, lactate; P, pyruvate; CSF, cerebrospinal fluid; MRI, magnetic resonance imaging; MRS, magnetic resonancy spectoscopy; NAA, N-acetyl L—aspartate; EMG, electropmyography; EEG, electroencephalography; ECG, electrocardiogram; OXPHOS, oxidative phosphorylation; mtDNA, mitochondrial DNA. Figure modified from Gorman et al 2016 and Haas et al 2008.

2.2.3.2 Magnetic resonance imaging (MRI)

MRI findings are typically not diagnostic alone and a normal finding does not rule out a mitochondrial disorder (4). Leigh disease is a neuroradiological diagnosis with bilateral symmetrical changes in brain stem and basal ganglia, and especially in putamina. MELAS is suspected when stroke-like lesions, not following vascular territories are detected (87). Leukoencephalopathy with brain stem and spinal cord involvement and high brain lactate (LBSL) caused by defective *DARS2* gene (mitochondrial aspartyl-tRNA synthetase) is an exception to this, as the brain MRI pattern is very specific (65, 88, 89).

In Alpers syndrome, occipital regions might show changes reflecting neuronal loss and gliosis. Hyperintensities are prominent in thalami and basal

ganglia when seizures are uncontrolled. Later, atrophy of basal ganglia and brain stem is visible with occasional cerebellar involvement. (39)

Magnetic resonance spectroscopy (MRS) uses the same signals as MRI to calculate the concentration of substances in the brain. In mitochondrial disorders, MRS might show high lactate peak in the central nervous system (CNS) (4). NAA is produced in mitochondria from aspartic acid and acetyl-CoA (90), and its quantity is used as a proxy of neuronal integrity (4). Large succinate peak may also be seen in patients with succinate dehydrogenase (CII) defects (91).

2.2.3.3 Tissue biopsy samples

Muscle tissue biopsies (or sometimes liver or heart biopsy samples) play an important role in the diagnosis of mitochondrial disorders, since they offer material for a wide variety of analyses. MtDNA heteroplasmy is preferably determined from post-mitotic tissue sample, since in some cases, heteroplasmy tends to fall in peripheral blood with age (92–94). Besides muscle, cells of urine sample or buccal mucosa swipe sample give a reliable heteroplasmy result. Biopsy samples offer material for a wide spectrum of other important analyses (see below).

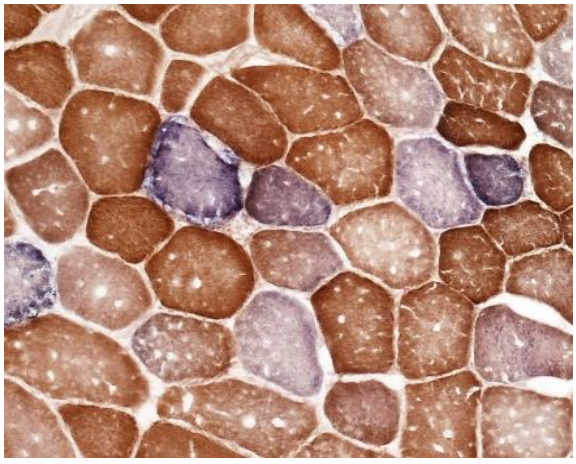


Figure 5. Immunohistochemical activity staining of muscle tissue from a patient with progressive external ophthalmoplegia (PEO). Cytochrome c oxidase (COX, in brown) weak or negative fibers colocalize with succinate dehydrogenase (SDH, in blue) abundant fibers. Figure: Anders Paetau

2.2.3.3.1 Histology

The most prominent histological hallmark for mitochondrial diseases in muscle are ragged-red fibers (RRFs), a sign of mitochondrial proliferation (95). The red appearance in Gomori trichrome staining is due to accumulation of abnormal mitochondria near plasma membrane of the cell. They are most common in disorders arising from dysfunctional mitochondrial translation or mtDNA deletions. Among children, RRFs are less common, and associate with specific gene defects, including TK2-gene defect (4, 96).

Histochemical activity analysis of RC complex IV (cytochrome c oxidase, COX) is routinely done simultaneously with complex II (succinate dehydrogenase, SDH) activity measurement. COX weak or negative fibers are indicative for a mitochondrial disorder, especially when the same fibers are abundant in SDH (Figure 5). SDH intensity reflects mitochondrial proliferation, which is induced as RC function diminishes. Complex II induction is possible even in mitochondrial translation defects, since CII is solely encoded by the nucleus.

Immunohistochemical stainings can be suggestive for genetic diagnosis, particularly when only one of the complexes is disrupted. This can indicate lack of assembly (defective *SURF1* in CIV defect) (97) or lack of a complex subunit.

2.2.3.3.2 RC enzyme activity analysis by spectrophotometry

Detection of a biochemical OXPHOS defect in a tissue mitochondrial preparation is done by giving it enzyme-specific substrates and measuring the enzyme activity relative to citrate synthase activity (4). A combined defect in complex activities might indicate a mitochondrial translational defect (CII not affected). Measuring coupled enzyme activities (I+III and II+III) may reveal defects in CoQ10 pathway, which can be verified by detecting decreased CoQ10 levels in tissue (4). Another assay, polarography, measures the amount of oxygen consumed by mitochondria when different substrates are given. This is less frequently available, because it requires a fresh tissue sample, but adds diagnostic sensitivity to standard RC analysis (98). Also, ATP synthesis capacity can be measured from isolated mitochondria.

2.2.3.3.3 Importance of tissue biopsy sample for research

Cells are excellent material, when pathogenicity of novel disease genes is being validated. Detection of a dysfunction in cells, and rescuing the phenotype with a wild type gene is good evidence for variant pathogenicity. Cells obtained from tissue samples can be cultured as such, or they can be stimulated to become induced pluripotent stem (iPS) cells. These cells have the potential to become any cell type of the organism. Regarding the high tissue-specificity of mitochondrial disorders, this approach might help to gain

access to cells from a tissue not eligible for tissue biopsy (i.e. brain). Cultured cells are also virtually an endless source of DNA. In addition, fibroblasts can be frozen and thawed for culture as novel diagnostic methods appear.

2.2.3.4 Genetic diagnostic testing

Finding a genetic diagnosis is the most exact way to achieve a definite mitochondrial disease diagnosis. Disease phenotype, model of inheritance and results of other assessments determine the best suitable genetic diagnostic test.

2.2.3.4.1 mtDNA sequencing

When inheritance pattern or disease phenotype strongly suggest a defect in the mitochondrial genome, mtDNA can be fully sequenced. The phenotype of the most common mtDNA point mutation, m.3243A>G, varies greatly hindering quick diagnosis (99). Still, single nucleotide analysis is appropriate when for example MIDD is suspected (Table 1).

2.2.3.4.2 mtDNA quantity

Real time quantitative PCR (qPCR) analysis reveals low mtDNA quantity (depletion). Such depletion in muscle is typically seen in diseases caused by mutations in *TK2, RRM2B, SUCLA2* and *SUCLG1* (86), the last three showing also central nervous system manifestation. A hepatocerebral form of mtDNA depletion syndrome is caused by defects in genes *MPV17, POLG, TWNK, DGUOK* and *TFAM* (86, 100). Such small panels of known causative genes enable targeted sequencing of the candidate nuclear genes.

2.2.3.4.3 Sequencing of nuclear candidate genes

Sequencing of candidate genes is possible if the phenotype is specific to a certain genotype. As mentioned earlier, this is the case in patients with LBSL caused by mutations in *DARS2*, where brain MRI is already diagnostic. Another example is patients with *TK2* mutation, where mtDNA depletion together with a severe dystrophy is highly suggestive for mitochondrial myopathy, even to the degree of a specific nucleotide change in the Finnish population (44). Genetic studies are then used only to verify diagnosis. In most cases, however, candidate disease genes are so many, that sequencing individual genes becomes too laborious and expensive (101). Currently, a mitochondrial gene panel can be even cheaper than sequencing of a one large gene (for example *POLG*).

2.2.3.4.4 Next generation sequencing (NGS)

Next generation sequencing is a massively parallel, high throughput sequencing technology developed during the past decade. It is highly efficient as hundreds or thousands of genes are sequenced simultaneously. Together with mtDNA, one can sequence for example 1) all nuclear genes known to cause a specific mitochondrial disorder (a custom-made panel) 2) all genes encoding a mitochondria-localized protein (MitoCarta 1.0, 1034 genes, (102)) 3) all coding regions of nuclear DNA (the Exome) 4) full genomic DNA including introns and non-coding regions (the Genome) (42).

NGS enables detection of a pathogenic variant in a single patient (103, 104), detection of novel disease genes (29, 105, 106) and detection of novel phenotypes in a known disease gene (107, 108).

2.2.3.5 Differential diagnosis

Differential diagnosis for mitochondrial diseases are multiple, demanding clinical pattern recognition.

Mitochondrial axonal neuropathy can resemble Charcot-Marie-Tooth disease, a neuropathy known to be caused by multiple gene defects. A mitochondrial neuropathy can also resemble inflammatory or autoimmune disorder. (109)

Central nervous system manifestations include epilepsy, encephalopathy, stroke-like episodes and ataxia. Epilepsy could be caused by an encephalitis or epilepsy syndrome and myoclonic epilepsy by ceroid lipofuscinosis or gluten encephalopathy. Mitochondrial epilepsy is typically accompanied with encephalopathy or other clinical features. Mitochondrial stroke-like episodes are distinguishable from ischemic stroke by the presence of a prodrome (migraine, flashing light, visual disturbance) and by the brain MRI: lesions caused by stoke-like episodes do not follow vascular territories and tend to change over time. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) causes strokes, dementia and migraine, but is caused by a NOTCH3 gene defect. Increased protein concentration in CSF is typical in CNS vasculitis. Purely cerebellar ataxia is rare in mitochondrial disorders and could be caused by toxic (alcohol), metabolic, inflammatory and degenerative causes. Fragile X associated tremor/ataxia syndrome (FXTAS) and vitamin E deficiency are non-mitochondrial causes of ataxia, whereas Friedreich's ataxia is a secondary mitochondrial ataxia.(109)

Mitochondrial PEO could be mixed with myasthenia gravis or oculopharyngeal muscular dystrophy, but in contrast to these, the severity of muscle weakness in mitochondrial PEO does not fluctuate and does not affect other facial musculature. Nemaline myopathy, myotonic dystrophy type 1 and desminopathy could also present as ptosis in addition to generalized muscle weakness. Limb-girdle syndromes and Dok-7 congenital myasthenic

syndromes typically cause induction of serum creatine kinase, which is rare in mitochondrial disorders (109).

An OXPHOS defect detected in muscle tissue might also be secondary. Spinal muscular atrophy (SMA), limb-girdle muscular dystrophy (LGMD), inflammatory myopathies and amyotrophic lateral sclerosis (ALS) are diseases where secondary mitochondrial defects are seen (110). Aging is associated with accumulation of point mutations and deletions in mtDNA and presence of COX-negative muscle fibers (111). Mitochondrial dysfunction might be present in for example copper-metabolism disorders, lysosomal disorders and peroxisomal disorders. Elevated blood lactate is a hallmark of mitochondrial disorders but present also in other inborn errors of metabolism.

Visual impairment could be caused by inflammation or structural pathologies as well as other genetic causes. Wolfram's and Usher's syndromes cause visual loss together with hearing deficiency. Susac's syndrome causes occlusion of microvessels in the brain, retina and inner ear via an autoimmune pathology. (109)

Mitochondrial parkinsonism presents typically with additional symptoms. The neurodegenerative features of Huntington's disease can falsely be interpreted as mitochondrial features although chorea is rare in mitochondrial disorders and typical in Huntington's disease. (109)

A multisystem disorder might suggest congenital glycosylation disorders or Marinesco-Sjögren syndrome. (67)

2.2.4 TREATMENT OPTIONS

Despite the first genetically verified mitochondrial disorder being described almost 30 years ago (112), there is currently no cure for these disorders (with the exception of CoQ10 deficiencies). However, a growing understanding of the mechanism of many mitochondrial disorders, has led to the discovery of therapies that improve survival and life quality.

Besides specific treatments, all patients should receive appropriate symptomatic and supportive management. Symptomatic medicine (avoidance of mitochondria-toxic drugs), individually designed exercise, medical equipment (cochlear implant, cardiac pacemaker), sufficient and personalized diet (avoid fasting), vaccination against infections and peer support are key factors in improving life quality of patients. (113)

2.2.4.1 Supplements

Many "mitochondrial cocktails" are used to treat a wide variety of mitochondrial disorders. Typically, they contain a variety of vitamins and supplements in high doses. There are few double blind, placebo-controlled studies evaluating their

efficacy and since these drugs also have possible harmful side effects, their wide usage cannot (yet) be recommended. (113, 114)

Coenzyme Q10 deficiency is treated with ubiquinol supplementation, a better absorbed, reduced form of CoQ10 (ubiquinone) (113). Intravenous administration of L-arginine might enhance outcome in patients with stroke-like episode due to MELAS (115).

It is worth noting that some mitochondria-affecting disorders that are not primary OXPHOS disorders, are treatable. Mitochondrial coenzyme deficiencies could be treated by supplementation of the coenzyme, such as thiamine and biotin in biotin-thiamine-responsive basal ganglia disease and biotin in biotinidase deficiency. (116)

2.2.4.2 Organ transplantation

Solid organ (heart, kidney, liver) transplantation in mitochondrial disorders was studied by Parikh et al in a retrospective study (113). After transplantation, they detected no acute deterioration of the underlying disease, but in most cases the disease progressed in other tissues with time. The best outcome was in patients with isolated liver manifestation (due to *DGUOK* mutation) where liver transplantation was essentially curative. Most of the liver failures in mitochondrial disease patients were due to valproic acid treatment of epilepsy, which is toxic for some patients with *POLG* mutations (68). The survival after transplantation was close to national averages, suggesting this as one of the treatment options after a careful risk-benefit assessment (113). Liver transplantation in Alpers-Huttenlocher disorder is not recommended given the fatal neurological course of the disease (117).

Allogenic haematopoetic stem cell transplantation has been suggested as a treatment for MNGIE disorder to restore thymidine kinase enzyme activity in blood cells and thereby reduce toxically high levels of thymidine and deoxyuridine in blood and other tissues. A retrospective analysis of 24 patients showed that if the donor HLA antigen match was 10/10 and transplantation age <30, outcome was beneficial. Thymidine kinase activity was restored quickly, but clinical improvement took time, even years. (118)

2.2.4.3 Preventive modalities

In case of a family being at risk to get a child with a mitochondrial disease, preventive modalities can be considered. Prenatal diagnosis from amniocentesis or chorionic villus sampling (CVS) is possible. Preimplantation genetic diagnosis is used to analyze genetic content of (a) very early stage embryo(s). In this, eggs of the mother are collected and fertilized *in vitro*. From a 3-day old embryo (8-cell stage), one of the blastomeres is investigated for the presence of known pathogenic mutation. A healthy embryo (or two) is implanted to the mother's uterus (Figure 6A). (119)

In case of heteroplasmic mtDNA point mutations, small insertions or deletions, using the above mentioned methods is not that straightforward. The heteroplasmy of mutated mtDNA in amniotic fluid, CVS and a blastomere is shown to be close to that of the whole fetus (33), but caution is warranted as unexpected exceptions might exist (120). An embryo, with heteroplasmy below a disease-specific threshold level for manifestation is deemed healthy, but as the threshold level is difficult to determine and termination of pregnancy with heteroplasmy near this level brings ethical concerns.

Unfortunately, these approaches are not applicable when there is a great risk for the fetus to have a high heteroplasmy level of mutated mtDNA (when mother has a high heteroplasmy). However, recent advances in technology and legislation have enabled a way to overcome this problem: mitochondrial replacement therapy. In this technique, the nucleus of the mother's egg (or pronucleus of the embryo) is transferred to a donated egg (or embryo) deprived of its nucleus (or pronucleus) (119, 121). The fertilized egg is then implanted to the mother's uterus (Figure 6B). Some maternal mitochondria carryover might happen, accounting only for a very low percentage of total mitochondrial content of the embryo. The developing fetus has genetic material from three individuals, donated mtDNA accounting for only <1% of the whole genetic material (119). This therapy was first approved in the UK in 2015 and has been used with promising results (122). This method brings some ethical concerns, discussed elsewhere (123–125).

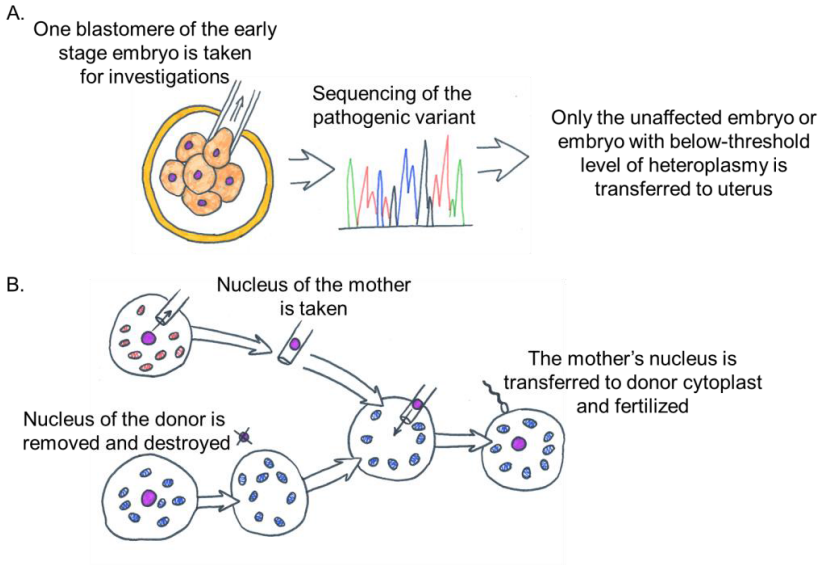


Figure 6. Simplified illustration of A) preimplantation diagnosis B) mitochondrial replacement therapy. Illustration: Kustaa Lehtonen

2.3 MOUSE MODELS FOR MITOCHONDRIAL DISORDERS

To study mitochondrial disease mechanisms, and to develop treatment for these devastating diseases, several genetically modified mouse models have been developed. This approach offers a great means to study disease in a controlled environment with homogeneous background.

An optimal disease model would have the same pathogenic mutation as patients and manifest with similar symptoms. This is not always possible, if the mouse protein is very different in its amino acid sequence or function. Sometimes a homologous mutation causes a different phenotype, as is the case in TK2 mice. Similar to humans, these mice show mtDNA depletion and OXPHOS deficiency, but in mice, the most severe symptoms were in CNS instead of muscle (126). Despite the challenges, the cellular processes still resemble each other and studies conducted using animal models often help to shed light on the disease pathogenesis as well as to develop treatment (127).

Introducing specific mutations to mtDNA has so far not been technically feasible, since introduction of DNA to mitochondria has not been possible (128). Disease models with mtDNA mutations have, however, been created by fusion of mutated mitochondria from somatic cell to an embryonic stem cell (ES cell). The resulting chimeric blastocyst is implanted to a pseudo-pregnant female. Developing pups are chimeric to mutant and wild-type mtDNA and mating of these females to wild-type males yields heteroplasmic offspring with varying phenotype (129, 130). Using a similar technique, Inoue et al (131) created Mitomice, a model that carries a heteroplasmic mtDNA deletion in all tissues and also transmits it to the offspring. This technique is challenging, since in some cases, there seems to be germline selection against the deleterious mutation in subsequent generations (130).

Editing nuclear genes is more straightforward. Genes can be silenced completely (called a gene knock out, KO), reflecting the situation in humans where a mutation causes for example a premature stop codon and preterm termination of protein synthesis, abolishing the function of the protein. Some gene deletions cause embryonic lethality (132, 133) and thus conditional methods are developed to circumvent such phenotypes by making tissue-specific KOs (as adipose tissue specific TFAM-KO (134)) or by making an inducible KO model (135).

Alternatively, a genomic construct with a mutation can be directed to the endogenous gene locus by homologous recombination, to generate a knock-in mouse model. Mutator mice have a defective POLG exonuclease activity as a critical aspartate residue is replaced with an alanine (136). These mice age prematurely due to somatic mtDNA mutations and dysfunction of mitochondrial RC.

The modified gene can also be injected to fertilized egg where it randomly integrates to genome. These transgenic mouse models express the mutated

gene as well as the wild type gene. This is how Tyynismaa et al created the Deletor mouse, a disease model with late-onset mitochondrial myopathy that readily replicates features of human PEO disease: accumulation of multiple mtDNA deletions and COX-deficient muscle fibers in skeletal muscle (137). These mice have a homologous dominant mutation in mitochondrial DNA helicase *Twnk* as Finnish patients with adPEO (138). Further characterization of this model revealed an AARE (amino acid response element) regulated transcriptional response, including induction of fibroblast growth factor 21 (*Fgf21*). FGF21 is a PPAR-α (peroxisome proliferator-activated receptor alpha) regulated (139) cytokine normally secreted by the liver (140). It is known to regulate glucose uptake in adipose tissue and to lower blood glucose and weight in diabetic and obese rodents (140). In Deletors, muscle mRNA expression of *Fgf21* increased along with COX-negative muscle fibers. Induction of expression was not detected in other tissues studied (liver, brain, fat), but has later been detected induced in Deletor heart (unpublished data). FGF21 is a secreted protein, also detected as elevated in Deletor plasma.

3 AIMS OF THE STUDY

The aims of this doctoral thesis are:

1) to develop and characterize new serum biomarkers for mitochondrial disease
2) to validate serum biomarkers and study the mechanism for biomarker induction
3) to discover novel genetic causes of mitochondrial disorders utilizing next-generation sequencing

4 MATERIALS AND METHODS

Detailed methods are given in articles I-III.

4.1 ETHICAL ASPECTS

These studies were approved by institutional ethics review boards and the declaration of Helsinki was followed. Participants (or guardians) gave written informed consents.

Animal procedures were performed according to protocols approved by the ethical boards for animal experimentation for each collaborating center. All experiments were done in accordance with good practice of handling laboratory animals and of genetically modified organisms.

Study participants were aware of the purposes of the study, magnitude and nature of possible discomfort caused and everyone was given a possibility to ask further questions about the study. None of this affected the treatment or patient car. All participants were given a possibility to quit participation at any point without having to name a reason.

We have minimized sampling harm by drawing a blood sample simultaneously with other diagnostic blood tests and doing it during anaesthesia when possible. Blood volume drawn at any one time has not exceeded 5% of body weight in children less than 10kg in weight.

4.2 PATIENT MATERIAL

Study subject inclusion for studies I and II are shown in Figure 7. In Suomalainen et al, pediatric control patients (n=18) were collected prospectively from Helsinki University Central Hospital as they attended the hospital for a non-mitochondrial disorder. In addition, 7 child controls and 49 adult controls were volunteers.

Patients with non-mitochondrial disorders or conditions studied in paper II were originally recruited for other studies and detailed information of these subjects is published elsewhere: ALS (141), cardiomyopathy (142), lymphoma (143), metastasized colorectal cancer (mCRC) (144), nonagenarians (145), primary biliary cirrhosis (PBC) (146), primary sclerosing cholangitis (PSC)(146), statin-induced myopathy (SM) (147). Fibromyalgia patients' samples were collected from pain outpatient clinics in Helsinki University Central Hospital, excluding patients with a diagnosed muscle disorder and/or severe psychiatric condition.

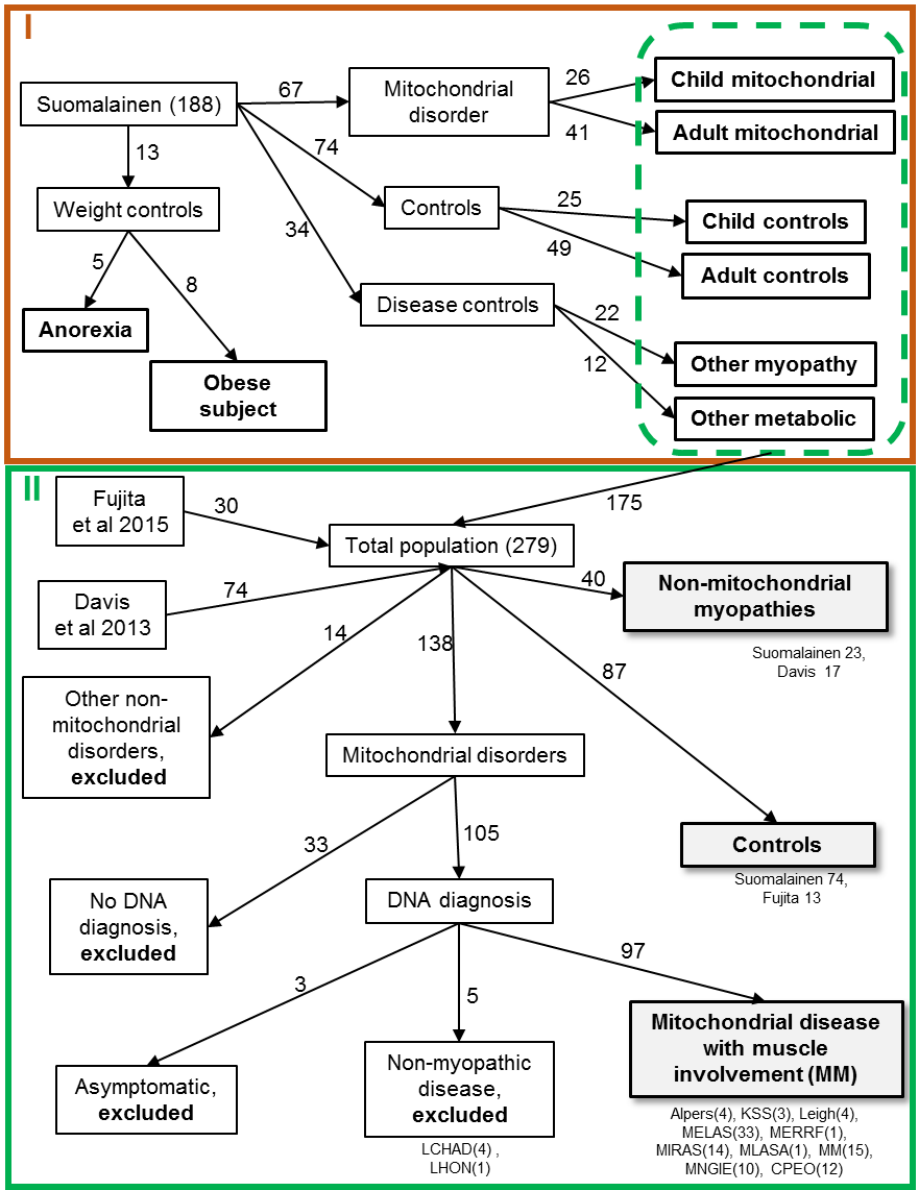


Figure 7. Flowchart of study subject inclusion in studies I (orange) and II (green). Text in bold highlights included subjects. Abbreviations: LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency; LHON, Leber's hereditary optic neuropathy; KSS, Kearns-Sayre syndrome; MELAS, mitochondrial encephalomyopathy, stroke like episodes and lactic acidosis; MERRF, myoclonic epilepsy with ragged-red fibers; MIRAS, mitochondrial recessive ataxia syndrome; MLASA, mitochondrial myopathy with sideroblastic anemia; MM, mitochondrial myopathy; MNGIE, mitochondrial neurogastrointestinal encephalopathy; CPEO, chronic progressive external ophthalmoplegia.

In study III, the index patient (II-2 of family I) was examined and treated in Helsinki University Central Hospital and parents gave written informed consent for participation in the study. Patients of family II were published originally elsewhere (148).

4.3 ANIMAL MODELS

Mitochondrial myopathy mouse models were analyzed for their serum FGF21 concentration and muscle *Fgf21* mRNA expression. Deletor mice have a homologous dominant mutation to Finnish patients with adPEO (in-frame duplication of amino acids 353-365 in Deletor corresponding to duplication of amino acids 352-364 in human) (138) in mitochondrial DNA helicase *Twnk.* This strain was generated in our own laboratory (149). K320E-Twinkle^{Myo} mice have a muscle-specific *Twnk* mutation (150). MitoMice carry a single heteroplasmic mtDNA deletion in tissues causing combined RC deficiency in muscle in high heteroplasmy (131). Ndufs4-KO mouse model causes a severe CI deficiency in muscle and brain (151). Mpv17-KO mice have mtDNA depletion and COX-negative fibers in muscle (152). Surf1-KO mice have CIV assembly defect, which is seen as decreased CIV activity in muscle (153).

4.4 ANALYSIS OF SERUM BIOMARKERS

Serum biomarker concentrations in patient serum/plasma were measured (by me) blinded for patients' group allocation with commercially available ELISA (FGF21: primarily BioVendor, Brno, Czech Republic; validation of the results with an independent ELISA kit from R&D Systems, Minneapolis, MN. GDF15: R&D Systems, Minneapolis, MN) according to manufacturers' instructions. Samples were diluted 1:2 (FGF21) and 1:4 (GDF15) unless concentration exceeded that of the highest standard. Serial dilution of concentrated samples was performed to obtain a more reliable result. All samples were analyzed in duplicates. Mouse samples were measured (by S. Forsström) with a Quantikine Mouse Fgf21 immunoassay (R&D Systems, Minneapolis, MN). Absorbance measurements were performed using a SpectraMax 190 absorbance microtiter plate reader (Molecular Devices, Sunnyvale, CA).

4.5 ANALYSIS OF MOUSE QUADRICEPS FEMORIS *FGF21* EXPRESSION

After total tissue RNA extraction, qPCR was run and *Fgf21* expression analyzed as described elsewhere (154) (by S. Forsström).

4.6 ANALYSIS OF MTDNA DELETION % IN MITOMICE

Determination of heteroplasmy level in Mitomice muscle samples was done with Southern blot as described elsewhere (155) (by S. Forsström).

4.7 EXOME ANALYSIS

Exome analysis was performed for patient II-2 in study III after mtDNA sequencing did not reveal any pathogenicity. Patient's total DNA was enriched by NimbleGen Sequence Capture 2.1 M Human Exome v1.0 array followed by sequencing with the Illumina Genome Analyzer-IIx platform. Variants were called according to variant calling pipeline used in Finnish Institute for Molecular Medicine (FIMM). This method revealed 58 685 SNVs with 53.23% of targets being covered at a minimum of 20-fold. These variants were then prioritized (by me and others) using a filtering strategy for likely disease causing variants (see results chapter 5.4.1).

NGS yields tremendous amount of genetic information that is considered classified. All data are stored encrypted and anonymously with only approved researchers having access to it.

4.8 PROTEIN KINETICS ASSAY

Mutated *FARS2* was expressed in *Escherichia coli* and protein function was measured in an enzyme kinetics assay *in vitro* as described elsewhere (156). Substrate binding and catalytic activity was measured for each mutation separately. Protein stability was examined by unfolding the protein and, after incubation, measuring the percentage of refolded protein with enzyme activity assay. These analyses were performed by our US collaborators (S.S. Yadavalli, E.M.Caruso and M. Ibba).

4.9 STATISTICAL ANALYSES

The normal cut-off value for serum FGF21 and GDF15 was determined as 95th percentile of a healthy control group. In study I, the cut-off limit for FGF21 was 200pg/ml, whereas in study II, it was 331pg/ml for FGF21 and 1014pg/ml for GDF15.

Patient data are expressed as mean (SD) (study I) or median (IQR) (study II). Mouse data variation is expressed as mean (SD).

Spearman's rank correlation analysis was used for analysing associations of S-FGF21 levels with continuous numeric variables (amount of COX-negative muscle fibers, body mass index (BMI), other biomarkers (including GDF15), age, deletion percentage in muscle of Mitomice, quadriceps femoris expression of *FGF21*). Nonparametric association was considered significant

if the r value was greater than 0.5 and $p<0.05$. In case r exceeded 0.5, linear regression was performed, and R^2 and P (capital p) value for goodness of fit are shown.

We used the Mann-Whitney U test (study I) or Kruskal–Wallis (human) or 1-way analysis of variance (ANOVA) (mice) followed by multiple comparisons test to compare FGF21 concentrations between patients and controls (study II).

To determine the diagnostic ability of biomarkers, we plotted the receiver operating characteristic (ROC) curves and calculated the area under the curve (AUC). Chi-square and Fisher's exact tests were used to calculate sensitivities, specificities and predictive values of biomarkers.

We did all statistical analyses with Stata (version 11.0) and PRISM 5 (study I) and PRISM 6 (paper II) (Graph Pad Software, La Jolla, CA). Two-sided p values were deemed significant at <0.05. Statistical analyses for study I were performed by K. Pietiläinen, for study II by me and S. Forsström and for study III by our US collaborators who performed the experiments.

5 RESULTS

5.1 FGF21 IS A NOVEL SERUM BIOMARKER FOR MUSCLE-MANIFESTING MITOCHONDRIAL DISORDERS (I)

Since FGF21 was found elevated in plasma of a mitochondrial myopathy disease model, Deletor (137) (see chapter 2.3), we decided to examine serum FGF21 concentrations in patients with mitochondrial myopathy. Samples were collected retrospectively from multiple centers to gather a large cohort of these rare disorders. We were able to sample 67 patients with an evident mitochondrial disease, 34 disease controls and 74 healthy controls. In addition, since FGF21 was earlier found to be positively associated with body weight (157), we examined five anorexic patients and eight obese individuals to control the potential effects of body weight in FGF21 serum levels in our patients.

5.1.1 S-FGF21 IS SPECIFIC TO MITOCHONDRIAL MYOPATHIES

Analysis of serum/plasma FGF21 concentration revealed significant induction of FGF21 in mitochondrial disorders, both in adults (mean 820pg/ml (SD 1151) vs healthy controls 76pg/ml (58), $p<0.0001$)) and children (1983pg/ml (1550) vs healthy controls 76pg/ml (58), $p<0.0001$). FGF21 concentration seemed to be higher in patients with mainly muscle phenotype (MELAS) than patients with mainly CNS manifestation (MIRAS). For MELAS, MIRAS and MNGIE (mitochondrial neurogastrointestinal encephalopathy), disease severity seemed to be associated with higher FGF21 value.

We analyzed a number of non-mitochondrial myopathies and other metabolic disorders to study specificity of FGF21 induction. No difference in S-FGF21 was detected in these groups, neither in adults nor children, when compared to healthy controls.

Despite the role of FGF21 in starvation (158–160), as well as its previously reported associations with BMI (157), patients with anorexia nervosa (BMI 15-17m^2) or obese individuals showed no difference in S-FGF21 compared to healthy controls. Association of BMI with S-FGF21 was not significant, neither in healthy controls nor in adult mitochondrial disease patients.

Liver is a known source of circulating FGF21 during starvation (158), and thus we asked whether the detected induction in serum could be explained by hepatopathy. Due to the retrospective nature of the study, liver function test results were not available from all patients, but in those patients that these measures were available (23 adults, 13 children), no evidence was found to support this possibility. However, muscle tissue samples were available from five adult mitochondrial disease patients, and the expression of *FGF21* was

determined. Similar to results in serum, MELAS patients with a considerable number of COX-negative fibers in muscle showed a greater induction in *FGF21* expression than MIRAS patients, who have mild muscle manifestation but prominent brain symptoms. As in Deletor mice, also in patients the number of COX-negative fibers correlated with S-FGF21 value, indicating that the sick muscle fibers, instead of liver, secrete FGF21 to blood.

5.2 FGF21 AND GDF15 ARE BOTH INDUCED IN MITOCHONDRIAL MYOPATHIES (II)

After our original study, others have replicated our results (62, 161, 162) as well as described another serum biomarker for mitochondrial diseases, GDF15 (163, 164). GDF15 is a member of the transforming growth factor beta (TGF-β) superfamily (165) and it is known to be secreted by liver, with expression being upregulated in liver tissue injury (166). Regarding mitochondrial diseases, GDF15 was found induced in a cell model of mitochondrial dysfunction, and increased amounts of this protein were then also detected in a small cohort of mitochondrial disease patients (MELAS, KSS, mitochondrial myopathy and mitochondrial disease) (163).

Table 3. *Accuracy and efficacy of diagnostic biomarkers in detecting mitochondrial myopathy from other clinically relevant differential diagnosis (non-mitochondrial myopathy, ALS (amyotrophic lateral sclerosis) and healthy control). Combination of FGF21 and GDF15 (in table rows 3 and 4) are only calculated for patients with both biomarkers measured (unpublished data). "FGF21 or GDF15" means that either one or both of the biomarkers is induced (vs. both normal) and "FGF21 and GDF15" means both biomarkers are induced (vs either one or both normal). *Results for lactate, pyruvate, L/P and CK obtained from paper I. Bolding indicates the two best values in each column. Statistics were performed with PRISM 7 (Graph Pad Software, LA Jolla, CA) and Fisher's exact test was used with two-sided p-value deemed significant at <0.05. Cut offs were 331pg/ml and 1014pg/ml for FGF21 and GDF15, respectively. Abbreviations: L/P, lactate to pyruvate ratio; CK, creatine kinase.*

Biomarker	sensitivity	95% confidence interval	specificity	95% confidence interval	positive predictive value	95% confidence interval	negative predictive value	95% confidence interval
FGF-21	67.3	57.8-75.8	89.3	83.2-93.7	82.6	73.3-89.7	78.2	71.3-84.2
GDF-15	**76.1**	64.5-85.4	86.4	77.4-92.8	81.8	70.4-90.2	81.7	72.4-89.0
FGF21 or GDF15	**83.1**	72.3-91.0	84.1	74.8-91.0	80.8	69.9-89.1	**86.1**	76.9-92.6
FGF21 and GDF15	56.3	44.1-68.1	**97.7**	92.0-99.7	**95.2**	83.8-99.4	73.5	64.6-81.2
Lactate*	63.0	47.6-76.8	92.8	83.9-97.6	85.3	68.9-95.1	79.0	68.5-87.3
Pyruvate*	73.9	51.6-89.8	87.2	74.3-95.2	73.9	51.6-89.8	**87.2**	74.3-95.2
L/P*	43.5	23.2-65.5	**100.0**	92.5-100.0	**100.0**	69.2-100.0	78.3	65.8-87.9
CK*	43.8	26.4-62.3	73.7	62.3-83.1	41.2	24.7-59.3	75.7	64.3-84.9

We performed a meta-analysis of muscle-manifesting patients of our previously published data (paper I), two other studies (the only ones available, listing individual S-FGF21 values and genetic causes) (161, 163), 17 new

genetically verified mitochondrial disease patients and 13 new non-mitochondrial myopathy (non-MM) patients (see Figure 7 for patient inclusion flowchart). We analyzed both biomarkers from our patients whenever possible.

Both markers were highly induced in patients with mitochondrial myopathy (MM) (FGF21 median 578pg/ml (IQR 289-1187) vs 66pg/ml (48-104) of controls; GDF15 2146pg/ml (1024-4312) vs 328pg/ml (235-474) of controls) and they also correlate to each other ($r=0.61$, $p<0.0001$). Sensitivities (proportion of true MM patients correctly identified as such) of FGF21 and GDF15 to detect a mitochondrial disease with muscle manifestation were 67.3% (57.8-75.8%) and 76.1% (64.5-85.4%), respectively (Table 3).

5.2.1 FGF21 AND GDF15 ARE INDUCED THE MOST IN MYOPATHIC DISEASES CAUSED BY A MITOCHONDRIAL TRANSLATION DEFECT

To study whether the trigger for biomarker induction is in the RC defect or somewhere upstream, we classified mitochondrial myopathy patients into three categories based on their genetic defect: 1) defects affecting mitochondrial translation (mt translation) 2) primary mtDNA deletions or defects leading to multiple mtDNA deletions (mtDNA deletions) 3) defects of RC subunits or assembly factors (RC structure & assembly). Moreover, we also analyzed quadriceps femoris muscle of mitochondrial myopathy mouse models (Deletor (149), K320E-Twinkle^{Myo} (150), MitoMice (131), Surf1-KO (153), Ndufs4-KO (151), Mpv17-KO (152)) for *Fgf21* mRNA expression and drew serum to measure circulating FGF21 concentration. These mice were categorized similarly to patients (albeit no translation group).

In human, both biomarkers were significantly ($p<0.0001$) elevated in translation and deletion groups compared to controls (675pg/ml (437-1504) and 347pg/ml (206-1062) vs 66pg/ml (48-104) for FGF21, 3092pg/ml (1844-4868) and 1520pg/ml (852-3403) vs 328pg/ml (235-474) for GDF15, respectively) (Figure 8 and 9). Both of these mutation types eventually lead to a translational defect.

Similarly in mice, defects causing mtDNA deletions caused a significant induction in FGF21 in serum (mean 1163pg/ml (SD 625) vs controls 379pg/ml (233), $p<0.0001$) as well as in quadriceps femoris (QF) *Fgf21* mRNA expression (10.9 times higher relative expression (SD 9.3), $p<0.0001$).

5.2.2 MUTATIONS IN RC STRUCTURAL SUBUNITS OR ASSEMBLY FACTORS DO NOT CAUSE BIOMARKER INDUCTION

Unlike in the first two groups described above, defects affecting RC structural subunits or assembly factors did not induce these markers to that extent. FGF21 was only mildly elevated (335pg/ml (54-604) vs 66pg/ml (48-104), $p<0.05$) while GDF15 showed no difference to controls (512pg/ml (348-1178) vs 328pg/ml (235-474), non-significant) (Figure 8).

In mice of the same group (Surf1-KO and Ndufs4-KO), serum FGF21 was not induced (335pg/ml (SD 96) vs 379pg/ml (SD 233), non-significant) and neither was QF *Fgf21* expression (0.9 times relative expression (SD 0.5), non-significant) (Surf1-KO, Ndufs4-KO and Mpv17-KO).

5.3 FGF21 IS NOT INDUCED IN NON-MITOCHONDRIAL DISORDERS (II)

As multiple differential diagnoses exist, it is important to validate the accuracy of these biomarkers to correctly identify MM from non-MM. For this, we analyzed 21 patients with a muscular dystrophy, 4 with a congenital myopathy, 16 with an inflammatory myopathy and 12 with other types of myopathies (17 of these published in Davis et al (161), see Figure 7).

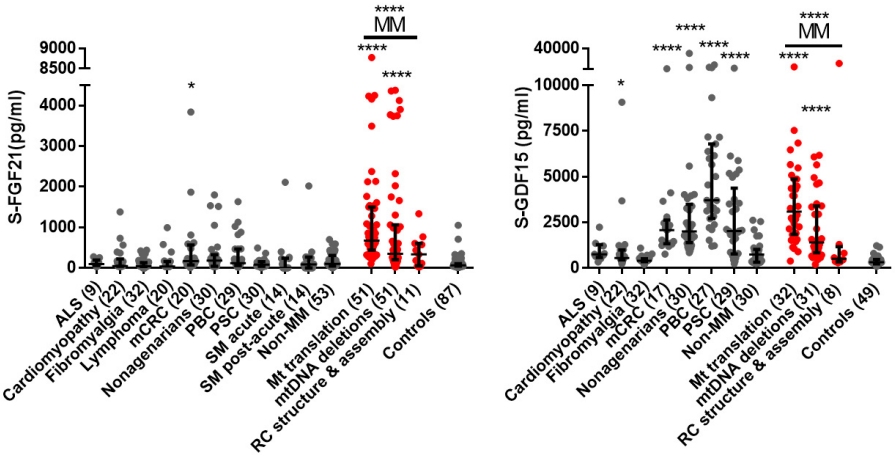


Figure 8. Serum biomarkers FGF21 and GDF15 in non-mitochondrial disorders and conditions, mitochondrial myopathies (MM) and controls. Patients with MM (in red) are categorized to three groups according to the genetic defect. ALS, amyotrophic lateral sclerosis; mCRC, metastasized colorectal cancer; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; SM, statin-induced myopathy in acute and post-acute phase; MM, mitochondrial myopathy; RC, mitochondrial respiratory chain. Each groups' median was compared to controls' median (Dunn's multiple comparisons test) and statistical significance of the difference is indicated as asterisks: * $p<0.05$, **** $p<0.0001$. Statistics were performed with PRISM 7 (Graph Pad Software, LA Jolla, CA). Note the discontinuous Y-axis. Parentheses indicate number of patients in each group.

Neither of the serum biomarkers were significantly different in non-MM than in controls (101pg/ml (46-309) vs 66pg/ml (48-104) for FGF21, 796pg/ml (344-1056) vs 328pg/ml (235-474) for GDF15). Among conditions typically examined in muscle clinics, specificities (proportion of non-MM patients

correctly identified as such) of these tests were 89.3% (83.2-93.7%) for FGF21 and 86.4% (77.4-92.8%) for GDF15 (Table 3).

Both biomarkers have also been studied widely in non-mitochondrial disorders. FGF21 has been associated with coronary heart disease (167), fatty liver (168, 169), type 2 diabetes (168, 170) and metabolic syndrome (170). Induction of GDF15 has been reported in for example ovarian cancer (171), chronic obstructive pulmonary disease (COPD) (172) and impaired fasting glucose (173).

We wanted to assess biomarker concentrations in non-mitochondrial diseases or disease traits that resemble mitochondrial diseases in some aspects. We chose patients/subjects with muscle involvement (ALS, cardiomyopathy, fibromyalgia, nonagenarians, SM), liver involvement (PBC, PSC and mCRC) or with a severe multi-organ disease (lymphoma, mCRC) to compare their biomarker levels to controls.

It turned out that besides mitochondrial myopathy, mCRC is the only group differing from controls in FGF21 concentrations (170pg/ml (75-566) vs 66pg/ml (48-104), $p<0.05$). However, GDF15 was induced in a wide variety of conditions, including cardiomyopathy (558 pg/mL (456–993), $p<0.05$)), mCRC (2,082pg/mL (1,332–2,625), $p<0.0001$), nonagenarians (2,007pg/mL (1,397–3,489), $p<0.0001$), PBC (3,706pg/mL (2,706–6,794), $p<0.0001$) and PSC (2,026pg/mL (774–4,371), $p<0.0001$) when compared to controls (328pg/mL (235–474)) (Figure 8).

5.3.1 PRIMARY MITOCHONDRIAL DEFECTS IN MUSCLE ARE ASSOCIATED WITH FGF21 INDUCTION

In line with the original findings (137, 174), results of paper II support FGF21 secretion by the muscle. In mice, serum FGF21 values associated with mtDNA deletion% in MitoMice (5)R^2=0.48, $p<0.05$) and QF *Fgf21* mRNA expression levels in mice with mitochondrial myopathy (R^2=0.43, $p<0.001$).

Interestingly, it seems that the induction requires a primary mitochondrial defect affecting mitochondrial translation or having mtDNA deletions. First, patients with a statin-induced myopathy showed no induction in S-FGF21 despite the CoQ10 lowering effect the drug has (175). Second, ALS is associated with secondary mitochondrial dysfunction (176), with no induction of S-FGF21. Third, according to the literature, nonagenarians (people >90 years of age) are shown to accumulate mtDNA deletions in tissues and a reduction of COX activity in muscle (111), still not showing induction in S-FGF21. Fourth, patients with inclusion body myositis (IBM), with considerable amounts of COX-negative fibers in muscle, had normal or almost normal S-FGF21 values (median 193pg/ml, IQR 57-319).

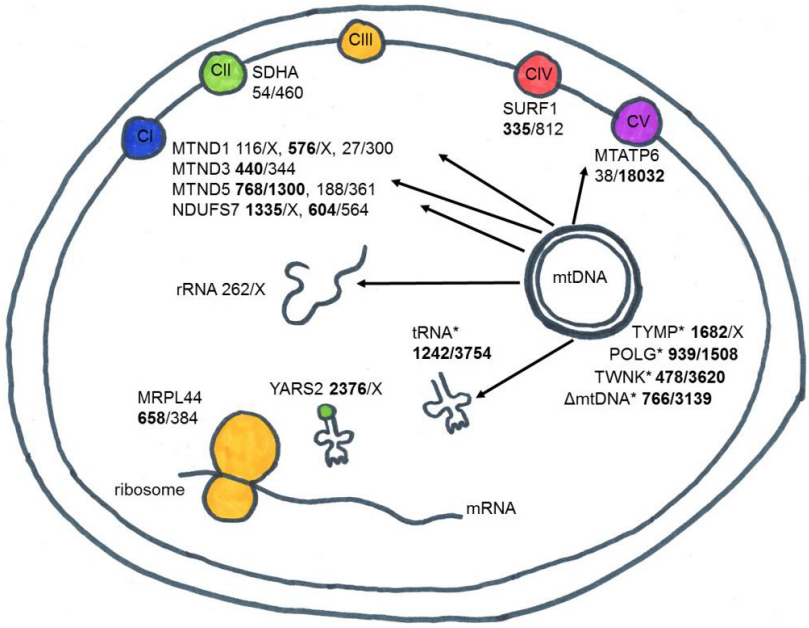


Figure 9. Simplified illustration of mitochondrion showing levels of serum FGF21/GDF15 according to genetic aetiology in patients with mitochondrial myopathy. Asterisk indicates median biomarker value (shown, if number of patient with this gene mutation is more than three). Bolding indicates values above normal range (331pg/ml for FGF21 and 1014pg/ml for GDF15). YARS2 protein catalyzes the attachment of tyrosyl to its tRNA. Abbreviations: ΔmtDNA, single large scale mtDNA deletion; mRNA, messenger RNA; tRNA, transfer RNA; rRNA, ribosomal RNA; CI-CV are OXPHOS complexes I to V. tRNA-category includes mutations in all tRNAs. HGNC approved gene symbols are used. X=not measured. Illustration: Kustaa Lehtonen

5.4 DEFECT IN *FARS2* IS A NOVEL GENETIC CAUSE UNDERLYING FATAL INFANTILE ALPERS ENCEPHALOPATHY (III)

At the initiation of this study, the genetic diagnosis of disorders had been undergoing a revolutionary increase in efficiency. Instead of single genes, we could now use massively parallel high throughput sequencing to analyze all protein-coding regions of the genome in a patient, by whole exome sequencing (WES). We decided to utilize this chance and used WES to search for a causative gene defect in a patient with fatal infantile Alpers encephalopathy.

Our index patient's disease started at the age of two days with treatment resistant myoclonic jerks. Blood and CSF lactate were elevated, suggestive of mitochondrial disease, while mtDNA sequence was normal. Plasma alanine, isoleucine and leucine were increased and she had generalized aminoaciduria. Histochemical activity of muscle COX was weak and

accumulation of lipids and glycogen were detected in muscle tissue. RC function was reduced: NADH: cytochrome c oxidoreductase activity was 114% of controls' mean, succinate: cytochrome c oxidoreductase 21%, succinate dehydrogenase 50% and COX 16% of controls' mean. Liver biopsy sample showed accumulation of glycogen, lysosomal iron and copper in addition to enlarged hepatocytes. Disease progressed rapidly, observed as generalized extensive brain atrophy in MRI at the age of 3 months. Brain MRS at three months showed elevated lactate as well as decreased NAA content supporting the diagnosis of a mitochondrial disease. The patient's myoclonic jerks continued and she had no psychomotor development. She died at the age of 8 months. Autopsy revealed severe cerebral atrophy, brain weighting only 30% of an age matched control. This patient was the second child of the family, the sister having died of a similar disease before the age of two.

5.4.1 EXOME SEQUENCING REVEALS PUTATIVELY PATHOGENIC VARIANTS IN *FARS2*

Exome sequencing of the patient DNA sample yielded 58 685 SNVs that were filtered according to the following criteria: The putatively pathogenic variant should be 1) in a protein-coding region of a gene 2) homozygous or compound heterozygous 3) predicted pathogenic by SIFT Genome tool 4) localized to mitochondria (according to MitoCarta 1.0 (31) 5) segregating with phenotype in the family 6) not present in population controls (400 Finnish control chromosomes and the 1000 Genomes database).

Identified variants in mitochondrial phenylalanyl-tRNA synthetase (mtPheRS, encoded by *FARS2*) were the only ones passing through this filter system. This protein catalyzes attachment of amino acid phenylalanine to its cognate $tRNA^{Phe}$ in mitochondria and is thus involved in mitochondrial translation, fitting nicely as a cause of the combined RC enzyme deficiency detected in index patient.

These variants were in exons 5 (c.986T>C, p.I329T) and 6 (c.1172A>T, p. D391V) (RefSeq NM_006567.3) of the *FARS2* gene and were conserved down to plants (Arabidopsis thaliana). We sequenced the protein-coding exons and exon-intron boundaries of *FARS2* in 82 patients with mitochondrial encephalopathy by Sanger sequencing but found no other patients with likely pathogenic mutations in this gene. The pathogenicity was strongly supported by another family with *FARS2* mutation (c.432A>G, p.Y144C), published elsewhere as a variant of unknown significance (VUS) (148). We combined our knowledge in the two research groups and did functional and structural validation of pathogenicity with the mutated proteins.

5.4.2 DETECTED VARIANTS DISRUPT PROTEIN FUNCTION

To model the mutated amino acids the crystal structure of mtPheRS protein was utilized, which revealed the motifs in which these amino acids are located

and allowed predictions of the functional consequences of the mutations to be made. First, p.I329T was predicted to widen the ATP binding pocket of the protein, decreasing the affinity of the enzyme to ATP. Second, both p.D391V and p.Y144C were predicted to affect the functional rotation mechanism of the protein that is needed upon substrate binding.

Our collaborators in USA (SS. Yadavalli and M. Ibba) expressed the wild type (Figure 10A) and mutated proteins in *E. coli* to study the effects of these mutations on protein function. They were able to show that p.I329T indeed decreases the ATP dependent amino acid activation efficacy of the protein (Figure 10B). The mutation p.D391V was found to have an increased K_m for phenylalanine, which may decrease mtPheRS aminoacylation activity (Figure 10C), whereas p.Y144C compromises the binding of $tRNA^{Phe}$ to mtPheRS hampering efficient enzyme function (Figure 10D).

In addition, capacity of refolding and thus reactivation of p.I329T and p.D391V single mutant proteins were disrupted compared to wild type protein, implicating a decrease in stability of the protein.

In concordance with these observations, decreased amounts of RC complexes were detected by BN-PAGE (blue native polyacrylamide gel electrophoresis) in brain and muscle of our index patient implying decreased translation of these proteins. CIV was reduced in both, and CI deficiency was evident in brain tissue. Fibroblasts did not manifest RC or translation deficiency.

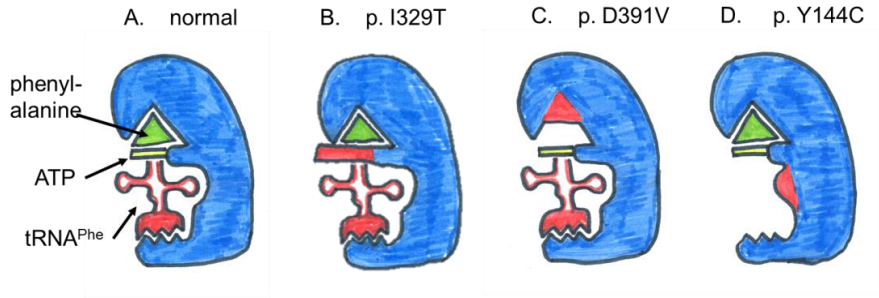


Figure 10. Schematic representation of mutated mtPheRS proteins. A) wild type mtPheRS showing normal localization of $tRNA^{Phe}$ (red), phenylalanine (green triangle) and ATP (yellow stick) B) mtPheRS with p.I329T mutation, with decreased capability to bind ATP in order to activate phenylalanine C) p.D391V mutation reduces the capacity of mtPheRS to bind to phenylalanine. D) p. Y144C mutant mtPheRS protein with decreased affinity to $tRNA^{Phe}$. Illustration: Kustaa Lehtonen.

6 DISCUSSION

We have discovered and studied a novel serum biomarker for muscle-manifesting mitochondrial disorders. It is the first new serum marker for these disorders since the measurements of lactate, pyruvate, their ratio and CK (all being in use already before 1980's (177–180)). In addition, we have studied another new serum biomarker, GDF15, and detected similarities as well as differences in the induction of these two biomarkers.

After our original report, induction of FGF21 in mitochondrial myopathies has been reported in several studies (62, 164, 181–183) and correlation with the amount of COX-negative fibers has been verified (181, 183), supporting our data that FGF21 is secreted by the muscle instead of liver or adipose tissue as in normal physiology . In all these studies, S-FGF21 exceeds the performance of the conventional biomarkers (lactate, pyruvate, their ratio, CK), strongly supporting the usage of this biomarker as a diagnostic tool.

GDF15 seems to be an even more accurate biomarker for mitochondrial disorders, demonstrated by an increased AUC in ROC analyses (paper II and two publications (164, 181)). Both GDF15 and FGF21 are similarly increased in mitochondrial translation defects (paper II) but not in defects directly affecting RC complex subunit or its assembly, thereby limiting their usage to a subset of RC disorders. However, we show (paper II) that unlike FGF21, GDF15 is also induced in many non-mitochondrial disorders and conditions, implying that this biomarker is less specific. FGF21 induction seems to require a primary mitochondrial disorder, which is not the case with GDF15. First, we show in paper II how GDF15 is induced in many non-mitochondrial diseases and conditions. Second, although GDF15 levels do not differ in controls and non-mitochondrial myopathies as groups (paper II and two publications (181, 182)), Ji X et al report occasional elevation of GDF15 in those non-mitochondrial myopathies that show secondary mitochondrial dysfunction (181). This is of particular interest, if this kind of difference could be used to direct further diagnostics and predict genetic aetiology.

Association of GDF15 with mitochondrial disease was first reported by Fujita et al in cell cybrids containing MELAS mutations, where GDF15 was induced in lactate-containing media (163). Before this, they had studied the same cells in normal medium (containing uridine) and reported an induction of ATF4 (activating transcription factor 4)(184), which is then shown to regulate both FGF21 (137) and GDF15 (185). Similar regulation of these biomarkers is possibly reflected in mitochondrial disease patients as significant correlation between these two biomarkers (164, 181, 182). Furthermore, they are both shown to reduce blood glucose levels in diabetic or obese mice (186, 187). In concordance, Nikkanen et al detected increased glucose uptake in skeletal muscle and heart of Deletors (the mouse model where induction of FGF21

was originally found), being a part of the stress response in mitochondrial myopathy (154).

These data indicate that FGF21 and GDF15 have similarities in their responses to mitochondrial dysfunction, but also differences, which is important information considering mitochondrial disease physiology. Detected differences in biomarker induction make it feasible to use them in combination: one is sensitive (GDF15), one is specific (FGF21). The usage of biomarkers in combination is supported by the higher sensitivity, specificity, positive and negative predictive values as compared to the use of biomarkers alone (Table 3, unpublished data). Presumably, even higher sensitivity could be achieved by measuring biomarkers repeatedly at different stages of disease course.

Accuracies of other diagnostic modalities are quite modest. Lactate and pyruvate are susceptible to false results due to a struggling child, postprandial sampling or poor sample handling (4). The sensitivity of lactate to detect mitochondrial disorders is quite unremarkable (63.0% (CI 47.6-76.8) in paper I) as also shown in Debray et al, where 52% of mitochondrial disease patients showed occasionally normal lactate values (83). Of tissue samples, skin fibroblasts are easiest to obtain, but they often lack the pathology seen in deep tissues (188). Among children, muscle biopsy sample is often normal, and negative findings do not rule out a mitochondrial disease (189).

In paper III, we detected *FARS2* mutations in infantile onset Alpers-Huttenlocher disease, adding a new gene to the list of three causative genes known at that time. The index patient had intractable myoclonic jerks, signs of hepatic disease and no psychomotor development fulfilling the diagnostic triad of Alpers-Huttenlocher syndrome. In addition, this patient had decreased NAA and elevated lactate in MRS, increased blood and CSF lactate, abnormal EEG and a compound defect in RC enzymes. Autopsy revealed neuronal loss, spongiosis and gliosis. All of these findings are typical for Alpers-Huttenlocher syndrome. This disease was severe, leading to death before the age of two in all four patients.

Detection of novel disease genes/variants needs further validation before establishing them as pathogenic. Variants detected in NGS need to be verified by Sanger sequencing (or other independent method), since NGS does make errors in areas of low coverage. Segregation of disease genotype with the phenotype is important to verify. Online databases contain a huge amount of important data for first-line validation. Conservation of the amino acid across species and variants' absence (or with recessive diseases, carrier frequency <1%)(190) in control populations is supportive of pathogenicity. It is worth noting that the control population should be carefully chosen since founder effects or haplotypes can interfere with interpretation. This was the case in patients with mutated *SARS2* (Table 4), where detected variants were absent in 212 Palestinian control chromosomes while in the particular village of patient's, carrier frequency was 1:15 (191).

Second-line validation requires patient samples, animal models (for

Table 4. The first human disease mutations described in mitochondrial aminoacyl-tRNA synthetases (aaRS). Year and method of detection, disease phenotype and functional validation of variant pathogenecity is listed to show a great variability in manifestation despite all impairing the same step of mitochondrial protein translation.

Gene	Full name	Protein	Year	NGS	Phenotype	OXPHOS defect	Number of patients	No of unrelated families	Type of functional evidence	Mutations	First reference
AARS2	alanyl-tRNA synthetase	mtAlaRS	2011	yes	infantile hypertrophic cardiomyopathy	CI, CIII, CIV in heart and brain	3	2	protein structure modeling	homozygous and compound heterozygous	Götz et al 2011
CARS2	cysteinyl-tRNA synthetase	mtCysRS	2014	yes	progressive myoclonic epilepsy, tetraparesis, visual and hearing impairment and cognitive decline	nd	2	one, consanguineous	mRNA splicing, protein structure modeling	homozygous, alternative splice site	Hallmann et al 2014
DARS2	aspartyl-tRNA synthetase	mtAspRS	2007	no	leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation (LBSL)	normal in fibroblast and lymphocytes	38	30	enzyme activity assay in *E.Coli* , detection of splice variant	compound heterozygous, alternative splice site in some	Scheper et al 2007
EARS2	glutamyl-tRNA synthetase	mtGluRS	2012	yes	leukoencephalopathy with thalamus and brainstem involvement and high lactate (LTBL)	CI, CIII, CIV reduced in muscle, CIII and CIV reduced in fibroblasts	12	11	nd	15 mutations, all compound heterozygous	Steenweg et al 2012
FARS2	phenylalanyl-tRNA synthetase	mtPheRS	2012	yes	rhabdomyolysis, developmental delay, pyramidal signs, haematological abnormalities, neuropathy, elevated lactate, TCA cycle intermediates in urine	nd	3	1	nd	homozygous	Shamseldin et al 2012
GARS*	glycyl-tRNA synthetase	GlyRS	2003	no	Charcot-Marie-Tooth disease type 2D and spinal muscular atrophy type V	nd	>18 affected family-members	5	nd	4 dominant mutations	Antonellis et al 2003
HARS2	histidyl-tRNA synthetase	mtHisRS	2011	no	ovarian dysgenesis and sensorineural hearing loss of Perrault syndrome	nd	5	1	mRNA splicing changed, reduced enzyme activity, yeast complementation study, fertility of *C.elegans* with *HARS2* mutation reduced	compound heterozygous, alternative splice cite	Pierce et al 2011
IARS2	isoleucyl-tRNA synthetase	mtIleRS	2014	yes	cataracts, growth hormone deficiency, sensory neuropathy, sensorineural hearing loss, skeletal dysplasia (CAGSSS) or Leigh syndrome	normal OXPHOS in fibroblasts	3 + 1 Leigh	3 related families and 1 family (Leigh)	reduced IARS2 protein in fibroblasts	homozygous, compound heterozygous in Leigh	Schwartzen-truber et al 2014

Abbreviations: NGS, next generation sequencing; OXPHOS, oxidative phosphorylation; No, number; nd, not determined or reported; tRNA, transfer-RNA; CI-V, OXPHOS complexes I to V; TCA, tricarboxylic acid cycle. *The cytoplasmic and mitochondrial variants of GARS are translated from alternative initiation sites. Association of this phenotype to mitochondrial function was not studied.

Gene	Full name	Protein	Year	NGS	Phenotype	OXPHOS defect	Number of patients	No of unrelated families	Type of functional evidence	Mutations	First reference
KARS**	lysyl-tRNA synthetase	LysRS	2010	no	Charcot-Marie-Tooth disease, developmental delay, self-abusive behavior, dysmorphic features, vestibular Schwannoma	nd	2	2	protein structure modeling, decreased enzyme activity, yeast complementation study	3 variants: 2 compound heterozygous and one dominant	McLaughlin et al 2010
LARS2	leucyl-tRNA synthetase	mtLeuRS	2013	yes	premature ovarian failure and hearing loss (Perrault Syndrome)	nd	2	one consanguineous, one not	protein structure modeling, yeast complementation study, *C.elegans* with *LARS2* mutation is sterile	homozygous, and compound heterozygous	Pierce et al 2013
MARS2	methionyl-tRNA synthetase	mtMetRS	2012	no	autosomal recessive spastic ataxia with leukoencephalopathy (ARSAL)	reduced CI activity in fibroblasts	54	38	reduced protein level, impaired translation and respiration, drosophila experiments	recessive, duplication and deletion	Bayat et al 2012
NARS2	asparaginyl-tRNA synthetase	mtAsnRS	2015	yes	mild intellectual disability and epilepsy or severe myopathy	CI and CIV reduced in muscle	2	one, consanguineous	decrease in charging capacity	homozygous, alternative splice site	Vanlander et al 2015
PARS2	prolyl-tRNA synthetase	mtProRS	2015	yes	Alpers syndrome, cardiomyopathy, macrosomy	CI and CIV reduced in muscle	1	1	nd	compound heterozygous, premature stop codon in one	Sofou et al 2015
RARS2	arginyl-tRNA synthetase	mtArgRS	2007	no	pontocerebellar hypoplasia	isolated or combined reduction of CI, CIII and CIV in muscle	3	one, consanguineous	production of abnormally short transcript and reduction in transcript in fibroblasts	homozygous	Edvardson et al 2007
SARS2	seryl-tRNA synthetase	mtSerRS	2011	no	tubulopathy, pulmonary hypertension and progressive renal failure in infancy (HUPRA syndrome)	a mild reduction in CI, CIII and CIV in muscle	3	2	impaired enzyme function	homozygous	Belostotsky et al 2011
TARS2	threonyl-tRNA synthetase	mtThrRS	2014	yes	axial hypotonia, limb hypertonia, severe psychomotor delay and high blood lactate	reduction in CI, CIII, CIV, CV in muscle, not in fibroblasts	2	1	diminished expression and protein quantity, decreased enzyme activity, rescue experiments	compound heterozygous	Diodato et al 2014
VARS2 ***	valyl-tRNA synthetase	mtValRS	2014	yes	childhood PEO, ptosis, ataxia	CI and CIV reduced in muscle	2	1	nd	compound heterozygous	Taylor et al 2014
YARS2	tyrosyl-tRNA synthetase	mtTyrRS	2010	no	myopathy, lactic acidosis and sideroblastic anemia (MLASA syndrome)	CI, CIII, CIV reduced in muscle, fibroblasts normal	3	one, consaguineous	reduced protein level, impaired mitochondrial translation, enzyme kinetics abnormal, protein structure modeling	homozygous	Riley et al 2010
WARS2	tryptophanyl-tRNA synthetase	mtTrpRS	2017	yes	intellectual disability, ataxia, muscle weakness, delayed psychomotor development, athetosis	nd	3	one, consanguineous	mislocation of protein, nonsense mediated decay, protein structure modeling	compound heterozygous	Musante et al 2017

Abbreviations: NGS, next generation sequencing; OXPHOS, oxidative phosphorylation; No, number; nd, not determined or reported; tRNA, transfer-RNA; CI-V, OXPHOS complexes I to V; PEO, progressive external ophthalmoplegia. **The cytoplasmic and mitochondrial variants of KARS are splice variants. KARS has also been detected in a mitochondrial OXPHOS disorder (Kohda et al 2016). ***possibly pathogenic

example *E. Coli* or yeast) or advanced computation. Protein crystal structure is needed to computationally predict the effect the mutation has to the protein. As not all crystal structures are resolved or homology to another species' protein is too little, this prediction is not always possible.

If the mutation causes a premature stop codon or an alternative splice site, the size and possibly also quantity of mRNA or protein should change (non-sense mediated decay, splicing, instability of protein) (192–196). The mutation can change a targeting sequence, causing mislocation of protein (197).

In silico molecular prediction of the structural changes and the functional consequences they have, help to plan further studies. The protein can be unstable (as mtPheRS was) or it can have a reduced activity (as mtPheRS had) *in vitro* or *in vivo*. Once a measurable phenotype is detected, a rescue by complementation assay is possible (192, 198, 199).

mtPheRS dysfunction could have been seen as a reduced mitochondrial translation rate or reduced OXPHOS activity (not CII since it is fully nuclear encoded). Activity of CI and CIV were reduced in muscle but lack of this phenotype in fibroblasts hampered their usage in functional validation. This is typical for defects in mitochondrial aaRSs that cause a wide spectrum of phenotypes. Disease causing mutations (all recessive) have now been detected in all 19 of them (versus only 8 in 2012), 11 of them found using NGS methods. Disease phenotype and type of functional validation used in the original reports of mutated aaRSs is shown in Table 4.

American College of Medical Genetics and Genomics (ACMG) has given guidelines on how to interpret pathogenicity of a VUS (200). According to these guidelines, finding three patients from two families with similar phenotype, the absence of detected mutations in the control population, the segregation of genotype with phenotype, and the results of the functional analysis of the mutated protein together highly indicate that *FARS2* is the pathogenic gene. This was a fourth gene reported to cause Alpers disease, currently also linked to mutations in *PARS2* and *NARS2* (201).

The tissue specificity of these disorders is interesting since they all catalyze the attachment of an amino acid to its cognate tRNA in mitochondrial protein synthesis. Translation impairment *per se* cannot explain this phenotypic variation, but the effect of defective incorporation of specific amino acids to the synthetized protein or other (unknown) functions of these enzymes could explain the difference. It is possible that the genome contains protective or harmful variations elsewhere, modifying the phenotype.

The success rate of WES to detect a pathogenic mutation in patients with an biochemical OXPHOS defect is superior (43-60%) (202) compared to the pre-NGS time (16,4%)(203). The best success rate is seen in with no previous genetic studies done (which permits the detection of mtDNA mutations also). There are multiple reasons for why not all genetic defects are found. The mutation can lie outside the targeted area (in introns or in genes not targeted),

the disease can be polygenic, the model of inheritance could be different than expected (*de novo* dominant instead of recessive) or the quality of sequence analyzed in that region can be poor. Detection of deletions and insertion requires additional computation, as these sequence reads do not align with reference sequence automatically. Low or no coverage in an area indicates possible deletion and this should be further assessed.

The inheritance model is assumed as recessive, if both parents are healthy. However, when studying single patient samples, it is possible that the mutation is *de novo* dominant. In our index patients, WES revealed 841 previously unknown variants in a coding region. 22 of them were homozygous and 131 were compound heterozygous. The remaining 688 were potential dominant mutations. Analysis of parental samples would greatly reduce the number of these variants, but since approximately 75 *de novo* mutations occur during individual's development (in the whole genome)(204), a trio analysis is not completely definitive.

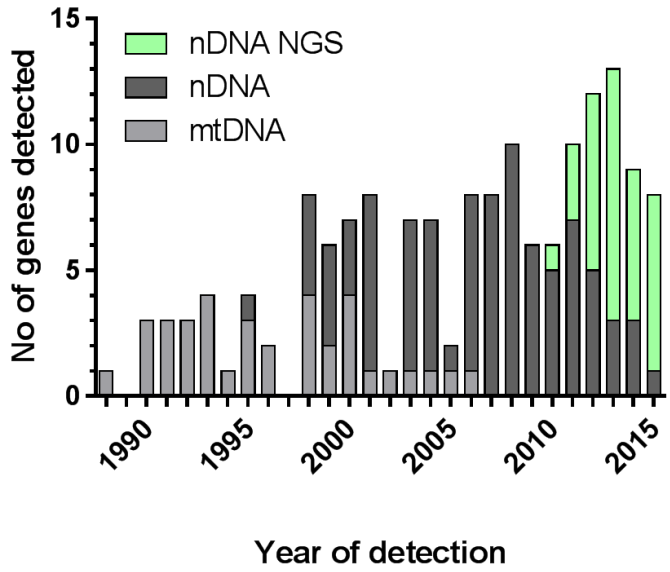


Figure 11. Number of novel primary OXPHOS disease genes detected each year (genes listed in Table 2). Green indicates nuclear genes detected with next generation sequencing (NGS) methods, gray indicates genes found using conventional methods. Graph drawn with PRISM 7 (Graph Pad Software, La Jolla, CA).

NGS brings some ethical concerns as the data could reveal something that was not looked for: genetic predisposition to cancer or a genotype of a later onset (possibly non-treatable) disease. In our studies, the NGS data filters are created to best serve our goal, finding a diagnosis for a mitochondrial disorder, leaving other types of variants unobserved. Genetic testing of pedigrees could

reveal the true (hidden) biological relationship of family members, knowledge that should not be transmitted to the family without their will. In 2013 (an update was published in 2015), ACMG created a list of genes whose pathogenic mutations should be returned to the clinician treating the patient (205, 206). A recommendation of the contents of the informed consent obtained from the patient before NGS was developed in 2013 by ACMG (207).

The usage and development of NGS technology during the last decade has enabled searching of the genetic aetiology also in single patients. The importance of NGS is also highlighted in Leigh disease, where nearly 30 out of 75 disease genes have been reported within the past 5 years (101). NGS techniques have enabled the sustained finding of novel genes causing primary OXPHOS disorders (Figure 11). Rapid expansion of the genetic library warrants re-analysis of NGS data yearly, as variants considered as VUS earlier, might have been reported as pathogenic later, confirming the diagnosis.

7 CONCLUSIONS AND FUTURE PROSPECTS

Heterogeneity of clinical manifestation of mitochondrial disorders in combination with an increasing number of possible genetic aetiology offer a complex task for clinicians, which is not simplified by inaccurate diagnostic tests available to date. Currently, diagnosis of a mitochondrial disorder relies on phenotype, histological evaluation of tissue sample, biochemical OXPHOS deficiency and a solid diagnosis is only received with genetic testing.

Finding a treatment for patients is the ultimate goal of our research. Before this, one must find a diagnosis, then to unravel the disease mechanism and only then that enables the search of tailored treatment development. Falling ill to a disease without a cure is a huge burden to the patient and family and uncertainties regarding disease only prolong the discomfort. Diagnosis brings clarity to situation and futile assessments can be avoided. Genetic diagnosis enables genetic counselling, which, in case of paediatric patients, is especially important if parents wish to have more children.

In the first publication (174), we characterized a novel serum biomarker for mitochondrial diseases, after decades of which there were no new biomarkers found. We show that it is most likely secreted by the diseased muscle fibers in mitochondrial myopathies, reflecting the disease stage in the muscle. The trigger for biomarker induction seems to be upstream of RC deficiency, most likely in mitochondrial translation. This marker is very specific for primary mitochondrial disorders.

In the second paper (208), we characterized and studied the mechanism of another new serum biomarker, GDF15. Similarly to S-FGF21, primary RC subunit defect or an assembly factor dysfunction causes a minor induction as compared to translational defects and mtDNA deletions. However, this biomarker is also induced in a variety on non-mitochondrial disorders, although not in the important differential diagnosis such as non-mitochondrial myopathies.

In the third paper (209) we used whole exome-sequencing to identify a novel disease gene, *FARS2*, causing severe infantile onset Alpers-Huttenlocher hepatoencephalopathy, a disorder previously only linked to *POLG, TWNK* (74) and *MTCO2* (75) mutations. Mutated *FARS2* hampers the function of its product, a mitochondrial phenylalanyl-tRNA synthetase (mt-PheRS), resulting in a mitochondrial translation defect. Mitochondrial aminoacyl-tRNA synthetases are a new group of genes underlying a wide variety of disorders, almost all of which have been first described within the last decade (Table 4).

Finding (and reporting) a novel disease gene, mutation or phenotype makes the finding of a second patient much more straightforward. Verifying the pathogenicity of a mutation in a tissue sample is sufficient to be done once,

and further patients do not need to donate any tissue for diagnosis validation (other than blood for the genetic testing).

Although FGF21 and GDF15 are the most accurate biomarkers for mitochondrial myopathies currently (Table 3), it is not yet known whether low biomarker values indicate normal/unspecific histology and RC enzyme activities in muscle suggesting that a muscle biopsy is not needed. To study this, we've started a multi-center collaboration with a prospective study set including all patients providing a muscle biopsy. In addition, it remains to be studied whether these biomarkers could benefit diagnosis of mitochondrial encephalopathies or if they could help to determine whether variants found with NGS are pathogenic.

During my thesis research, NGS methods have become more readily available while prices have sunk. Currently, whole genome sequencing (3.3 Gbp) is essentially as expensive as whole exome sequencing (30 Mbp) was at the time of publication of my third article (2012). Supposedly, the course will remain similar making it possible that NGS would be cost-effective as a first line diagnostic test within the next decade(s). Increasing knowledge of disease-related genes, and frequencies of variants found in healthy people, makes the detection of mutations feasible. Possibly, within the next decade, NGS data filtering and interpretation could even be (partially) automated.

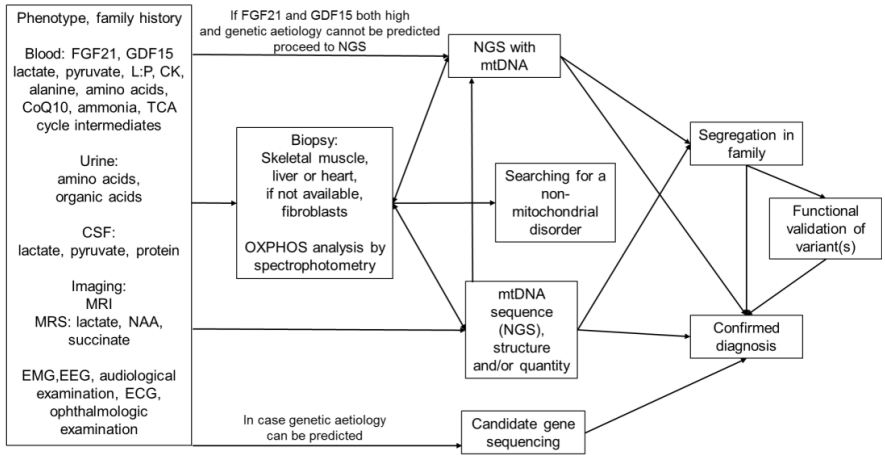


Figure 12. Suggestion for an updated diagnostic flowchart. Serum FGF21 and GDF15 should be included as first line diagnostic tests. Positive predictive value for the combination of these biomarkers is so high that rapid progression to gene sequencing is advised. NGS techniques should replace the traditional sequencing methods if a defective gene cannot readily be predicted based on phenotype. Abbreviations: L, lactate; P, pyruvate; CSF, cerebrospinal fluid; MRI, magnetic resonance imaging; MRS, magnetic resonancy spectoscopy; NAA, N-acetyl L—aspartate; EMG, electropmyography; EEG, electroencephalography; ECG, electrocardiogram; OXPHOS, oxidative phosphorylation; mtDNA, mitochondrial DNA; NGS, next generation sequencing. Figure modified from Gorman et al 2016 and Haas et al 2008.

Recently, Nikkanen et al analyzed over 100 metabolites in patients with mitochondrial myopathy and IOSCA (infantile onset spinocerebellar ataxia) due to *TWNK* mutation and reported genotype-specific metabolite fingerprints (154). Future research will show whether this type of metabolic profiling has the potential to become the next generation biochemical analyses for mitochondrial disease diagnosis.

In conclusion, we suggest that serum FGF21 and GDF15 should be used as front line diagnostic tests when a mitochondrial myopathy is being suspected before proceeding to muscle biopsy. Costs (blood sample draw, ELISA analysis) and harm of these analyses are minor compared to the benefits, supporting the incorporation of these biomarkers to the spectrum of obtainable tests in tertiary care diagnostic laboratories. Despite them not detecting mitochondrial RC subunit and assembly factor defects, their positive predictive power in combination leaves almost no doubt of the genetic aetiology. Low biomarker values do not out rule a mitochondrial disorder and thus expert clinical interpretation is needed. We advise to rapidly proceed to massively parallel NGS instead of sequencing candidate genes one by one especially if both biomarkers are induced (Figure 12).

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